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Protein Synthesis and the Onset of Intracellular Bacteriophage Growth

I. Stabilization of the Infecting Phage to ^{32}P Decay†

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(*Received 28 May 1960*)

Highly ^{32}P-labeled T2 phages become refractory to inactivation by radioactive decay within the first 10 min of their intracellular growth in the *E. coli* host cell. This stabilization against decay does not occur if infection takes place in the presence of chloramphenicol. If chloramphenicol is added to the infected culture only after the 4th min of intracellular growth of labeled T2, then stabilization is arrested at a stage intermediate between full sensitivity and complete resistance to decay. Hence protein synthesis is required not only for the start but also *throughout* the course of the stabilization process. Protein synthesis is also required for the intracellular decay stabilization of phage T1, whose DNA contains the same purine and pyrimidine bases as the bacterial host. It is unlikely, therefore, that the protein required for the stabilization process represents only the *de novo* enzymes of the metabolism of phage-specific DNA constituents. It seems more probable that, depending on the mechanism underlying the stabilization process, this protein either "activates" the replication of individual viral DNA molecules or represents the viral gene products.

1. Introduction

Highly ^{32}P-labeled bacteriophages are inactivated by the decay of the radioactive atoms contained in the phosphate diester bonds of the polynucleotide chains of the viral DNA (Hershey, Kamen, Kennedy & Gest, 1951; Stent & Fuerst, 1955). It is possible to examine the decay sensitivity of vegetative phages derived from infection of bacteria with ^{32}P-labeled viruses by allowing intracellular phage development to proceed for various times and then freezing and storing the infected complexes at $-196°C$. In this way, the slow radioactive decay can occur over periods of days and weeks at various well-defined stages of virus reproduction. In such experiments, infective centers frozen at the very outset of intracellular phage growth are inactivated by radioactive decay at nearly the same rate as the free, extracellular virus particles. Infective centers frozen at later times, however, are less sensitive to decay than the free virus, and when phage growth has been allowed to proceed for more than 10 min the infective centers are no longer inactivated at all by radioactive disintegrations occurring in the DNA molecules introduced into the host cell by the parental phage particles. A gradual *stabilization* to decay, therefore, takes place at the early stages of intracellular phage development. (Stent, 1955; Stent & Fuerst, 1956, 1960.)

In the present work, we have examined the relation of protein synthesis to this radiostabilization of the intracellular, vegetative phage. In particular, we have attempted to study the role of the "early" protein which is synthesized contemporaneously with the stabilization reaction in the virus-infected bacterium, since the

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replication of the DNA of coliphages T2 and T4 (hereafter referred to collectively as "T-even" phages) cannot commence before some protein synthesis has occurred. Once this "early" protein *is* present, viral DNA synthesis can proceed while further protein synthesis is inhibited by the presence of the antibiotic chloramphenicol (Burton, 1955; Melechen, 1955; Tomizawa & Sunakawa, 1956). Extensive experiments have shown that the DNA synthesized in T-even infected bacteria brought into contact with inhibitory concentrations of chloramphenicol later than the 10th min of phage growth is "functional", in the sense that this DNA ultimately appears as the genetic substance of infective progeny viruses formed after the removal of the antibiotic (Hershey & Melechen, 1957; Tomizawa, 1958). Some of the components of the essential "early" protein have already been discovered; they represent a number of new enzymes which are not present in the bacterium prior to infection and whose synthesis is induced at the outset of the growth of T-even phages. These enzymes are all concerned with the metabolism of 5-hydroxymethylcytosine (HMC) and other specific components of the T-even phage DNA (Flaks & Cohen, 1957; Kornberg, Zimmerman, Kornberg & Josse, 1959; Somerville & Greenberg, 1959; Keck, Mahler & Fraser, 1960). It is evident, therefore, that the replication of viral DNA cannot get under way in the infected cell until all of those enzyme proteins have been formed which are necessary for the synthesis of the essential polynucleotide building blocks.

The experiments presented here show that protein synthesis is indeed required for the intracellular stabilization of labeled phages to ^{32}P decay. The results indicate, however, that the enzymes of the metabolism of phage-specific DNA constituents are probably not the only "early" protein involved in this process, since protein synthesis is required not only at the start but throughout the course of the stabilization reaction. This requirement exists also for the attainment of the ^{32}P-decay resistant state of phage T1, whose DNA composition is indistinguishable from that of the host bacterium and whose replication would not, therefore, be thought to depend on the appearance of any new enzymes of DNA metabolism.

2. Materials and Methods

Phage strains. Coliphages T1 and T2 (H).

Bacterial strains. Escherichia coli, strain R2 (Hershey, Dixon & Chase, 1953) was the host organism in the growth experiments to be described. The term *standard suspension* of R2 bacteria refers to a culture grown to a density of 5×10^8 cells per ml. in tris-glucose medium, centrifuged, washed once, resuspended in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, and starved for 1 hr. Stocks of highly ^{32}P-labeled phages were grown on *E. coli* B/*r* (Witkin, 1951). For plaque assay, tryptone agar plates were seeded with *E. Coli* BB (McFall & Stent, 1958).

Temperature. Unless indicated otherwise, the temperature of incubation was 37°C in all of the growth experiments reported here.

Media. The casamino acid-glycerol medium was that devised by Fraser & Jerrel (1953). *Tris-glucose medium* was prepared according to Hershey & Melechen (1957), except that it was reinforced by addition of 0·05% casamino acids DIFCO. The same medium without glucose or casamino acids was used as adsorption buffer in experiments involving T2, and will be referred to as *tris-buffer*. The adsorption medium for phage T1 contained per liter of water: 0·15 g $MgSO_4.7H_2O$, 1 ml. 1% gelatin, and 0·5 ml. N-$CaCl_2$. The composition and preparation of *H medium* of high ^{32}P specific activity have been previously described (Stent & Fuerst, 1955). In the preparation of highly ^{32}P-labeled T1, however, the H medium did not contain any NaCl, and will be referred to as *low-salt H medium.*

Radiochemical techniques. The procedure of growing highly ^{32}P-labeled T2 phage stocks has already been described (Stent & Fuerst, 1955). For the preparation of *nutrient free*, highly labeled phage stocks, this technique was modified in the following way: the

infected culture of B/*r* growing in highly labeled H medium was centrifuged 2 hr after inoculation with phage and the bacterial pellet resuspended in tris-buffer. Immediately after resuspension, the intracellular, mature progeny phages were released by lysing the bacteria with chloroform (Fredericq, 1952). The phage stock was then freed from bacterial debris by centrifugation and from dissolved chloroform by gentle bubbling for a few minutes. Highly ^{32}P-labeled stocks of T1 were made according to essentially similar procedures, except that low-salt H medium was employed and that the B/*r* cells were incubated in the highly ^{32}P-labeled growth medium for 1 hr prior to their infection with T1 phage.

The synthesis of nucleic acid in phage-infected cultures growing in ^{32}P-labeled media was measured by trichloroacetic acid precipitation of the culture and Schmidt-Thannhauser fractionation of the cellular contents followed by counting of the radioactivity of the various fractions, in a manner similar to the procedure described by Hershey & Melechen (1957). The amount of phosphorylated material so measured is presented here in terms of "phage units" per cell, ϕ, reckoned from the following expression

$$\phi = \frac{c}{n.A_0.b.p}$$

where c is the total number of ^{32}P counts per min measured in the DNA fraction of the sample, n the number of counts/min/mc, A_0 the specific activity in mc/mg P of the ^{32}P-labeled growth medium, b the number of infected bacteria contained in the sample and p the amount of phosphorus per phage particle: for T1, $p = 0{\cdot}7 \times 10^{-14}$ mg (Stent & Fuerst, 1955); for T2, $p = 2 \times 10^{-14}$ mg (Hershey *et al.*, 1953).

Chloramphenicol. Crystalline chloramphenicol was obtained from Parke, Davis & Co.

3. Experimental

(a) *^{32}P decay sensitivity during intracellular phage growth*

(i) *Phage T2*

It was shown by Tomizawa & Sunakawa (1956) that the gradual decrease in the ultraviolet light sensitivity of T2-infected bacteria, first observed by Luria & Latarjet (1947), does not occur if infection is carried out in the presence of chloramphenicol, an antibiotic which inhibits the synthesis of protein in *E. coli* (Wisseman, Smadel, Hahn & Hopps, 1954). Protein synthesis, therefore, is necessary for the onset of those reactions which lead to the low intrinsic ultraviolet sensitivity of the phage-bacterium complex. In an unpublished experiment, Tomizawa also infected bacteria with highly ^{32}P-labeled T2 phages in the presence of chloramphenicol and noticed that likewise no reduction in ^{32}P decay sensitivity of the infective centers would develop as long as protein synthesis was inhibited by the presence of the antibiotic. We have confirmed this latter result by means of an experiment presented in Fig. 1. It may be seen there that the normal stabilization to ^{32}P decay has indeed taken place in bacteria infected with highly ^{32}P-labeled T2 phages in the absence of chloramphenicol, since the phage-producing ability of bacteria in which intracellular phage growth has been allowed to proceed for 11 min before freezing and storage is no longer destroyed by subsequent radioactive disintegrations. On the other hand, it is apparent that little or no stabilization occurs in similarly infected bacteria to which 25 μg/ml. chloramphenicol is added *before* the onset of intracellular growth, since the phage-bacterium complexes incubated for 40 min in the presence of the antibiotic have remained nearly as sensitive to ^{32}P decay as they were at the time of infection. Finally, it can be seen that in bacteria infected with the labeled T2 in the absence of the antibiotic but brought into contact with 25 μg/ml. chloramphenicol 4 min *after* the onset of intracellular phage development, at a time when the stabilization to ^{32}P was already under way, further increase in radioresistance is arrested by the addition of the antibiotic. Evidently 80% of the infective centers of a portion of this culture frozen

after further incubation for 36 min are still inactivated almost as rapidly as the complexes frozen at the 4th min. Some 20% of the infective centers, however, appear to be inactivated at a somewhat slower rate, corresponding roughly to the sensitivity of complexes in which phage development has proceeded for 6 to 7 min. This heterogeneity in sensitivity is probably due to the spread in time required for penetration of the antibiotic into, and establishment of its inhibitory effect in, different members of the population of infected cells.

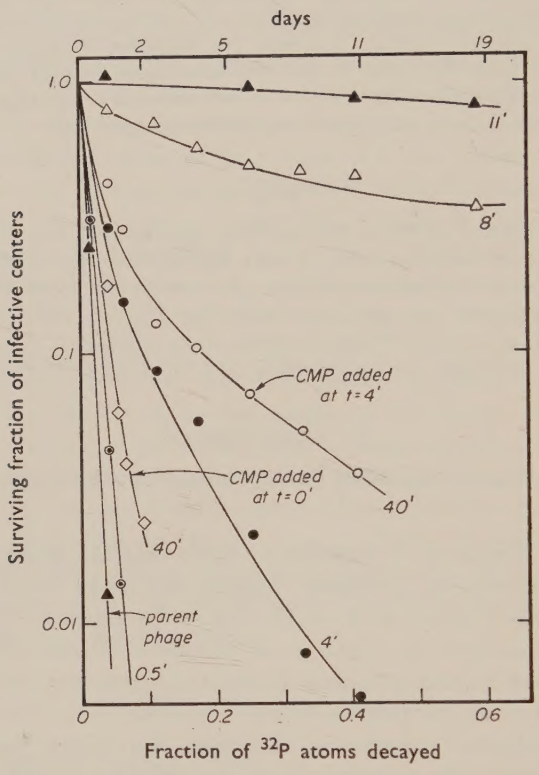

FIG. 1. The effect of 25 μg/ml. chloramphenicol (CMP) on the intracellular decay sensitivity of ^{32}P-labeled T2 phage. The number of minutes for which intracellular phage growth has been allowed to proceed before freezing and storage of the infected bacteria is indicated on each curve.

Procedure: A standard suspension of R2 bacteria was infected with $7{\cdot}0 \times 10^8$ viable phage particles per ml. of a nutrient-free radioactive stock of T2 labeled at a specific ^{32}P activity of 1600 mc/mg P. Adsorption of the phage was allowed for 5 min; colony survival counts of the infected culture at this time indicated an average multiplicity of infection of 1·2 phages per cell. A sample of the infected culture was then diluted 100-fold into tris-glucose medium containing 25 μg/ml. chloramphenicol and anti-T2 serum (k = 6) (growth tube no. 1) and glucose and casamino acids added to the remainder of the infected culture (growth tube no. 2). 4 min after the start of phage growth, a sample was removed from growth tube no. 2 and diluted 100-fold into tris-glucose medium containing 25 μg/ml. chloramphenicol (growth tube no. 3). At various times, portions of each growth tube were diluted into ice-cold glycerol-casamino acid medium. Numerous samples of each of these portions were then frozen and stored at — 196°C. Samples of the ^{32}P-labeled parental T2 stock were also diluted into glycerol-casamino acid medium and similarly frozen and stored. From day to day, one of the frozen samples of each portion was thawed and plated at once for assay of the number of surviving infective centers.

These results thus confirm the observations of Tomizawa, and show also that the synthesis of protein is not only required for allowing the *onset* of the evolution of ^{32}P-decay resistance, but that protein synthesis must proceed *throughout* the course of those reactions which render the phage-bacterium complex refractory to inactivation by radioactive disintegrations in the DNA of the infecting virus.

(ii) *Phage T1*

The synthesis of an "early" protein appears to be required not only for DNA replication of the T-even phages but also for DNA replication of phage T5, whose DNA is composed of the same four purine and pyrimidine bases as that of its *E. coli* host, though in quantitatively different proportions (Pfefferkorn & Amos, 1958; Crawford, 1959). Similarly, the replication of the genome, and hence, by inference, of the DNA, of phage λ cannot get under way without prior protein synthesis, even

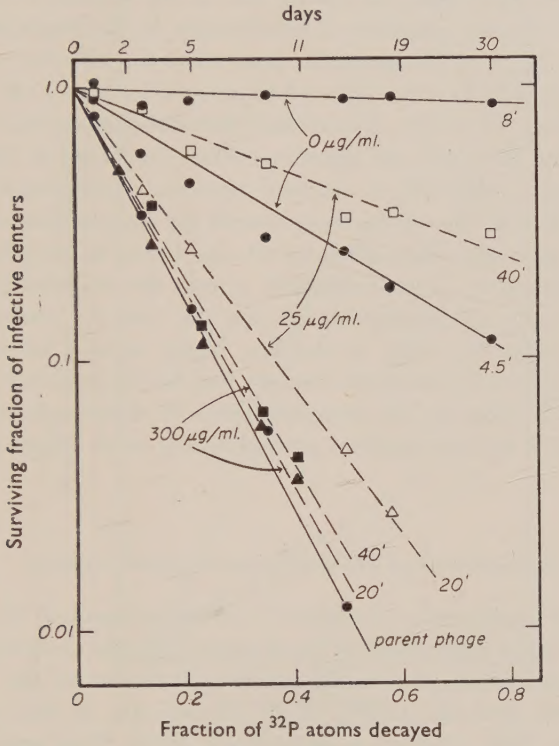

FIG. 2. The effect of various concentrations of chloramphenicol (CMP) on the intracellular decay sensitivity of ^{32}P-labeled T1 phage. The number of minutes for which intracellular phage growth has been allowed to proceed before freezing and storage of the infected bacteria is indicated on each curve.

Procedure. A standard suspension of R2 bacteria was centrifuged and resuspended in twice its original volume of T1 adsorption medium containing 300 μg/ml. chloramphenicol, 0·1% glucose and 0·05% casamino acids. After incubation of this culture for 5 min neutralized KCN was added to a final concentration of 0·01 M and the suspension infected with 2×10^8 viable T1 particles labeled at a specific ^{32}P activity of 200 mc/mg P. Adsorption of the phage was allowed for 8 min, and the infected bacteria centrifuged, washed once and resuspended in ice-cold tris-buffer. Intracellular phage growth was then initiated by diluting portions of the infected bacterial suspension into warm tris-glucose medium, containing either no chloramphenicol, or 25 μg/ml. chloramphenicol, or 300 μg/ml. chloramphenicol. At various times samples of each of these three growth tubes were diluted into ice-cold glycerol casamino acid medium.

though here the composition of the viral DNA is indistinguishable from that of the *E. coli* bacterium (Thomas, 1959). These findings seem, at first glance, surprising, since one might have thought that in these systems the host cell already contains all the necessary metabolic equipment for making the building blocks of the phage DNA. We have investigated, therefore, whether the decay stabilization process also requires prior protein synthesis in the intracellular growth of ^{32}P-labeled T1 phage, whose DNA, like that of λ, is analytically indistinguishable from that of its *E. coli* host (Creaser & Taussig, 1957). It seemed quite possible that stabilization of T1 might occur

without prior protein synthesis, since it had been reported that DNA synthesis does proceed in *E. coli* cells infected with T1 phages in the presence of chloramphenicol (Crawford, 1957). However, in view of the very fact that the compositions of T1 and *E. coli* DNA are indistinguishable by present-day chemical analyses, it is not possible to conclude from this observation that the post-infection DNA synthesized under these conditions is actually viral, and not host DNA.

The results of such a decay stabilization experiment of ^{32}P-labeled T1 phage are presented in Fig. 2. It is evident, first of all, that in the absence of chloramphenicol, T1-infected bacteria become refractory to inactivation by disintegrations of ^{32}P atoms incorporated in the DNA of the parental virus, since the infective centers in which intracellular phage growth has been allowed to proceed for 8 min are seen to be completely stable to decay. Secondly, it is evident that chloramphenicol *does* inhibit the stabilization reaction. Although the infective centers incubated in the presence of 25 μg/ml. chloramphenicol gradually do become more and more refractory to inactivation, this concentration of the antibiotic so retards the process that some decay sensitivity still remains even after incubation for 40 min. It can be seen, furthermore, that the presence of 300 μg/ml. chloramphenicol arrests the stabilization process completely, since even after incubation for 40 min the infective centers are nearly as sensitive to inactivation by decay as the free, highly labeled parental T1 virus. It thus appears that protein synthesis is necessary also for the intracellular decay stabilization of phage T1, although the concentrations of chloramphenicol required for complete inhibition of the stabilization process appear to be higher here than in the case of phage T2.

(b) *Synthesis of DNA in phage-infected bacteria*

The very gradual stabilization of labeled T1 in the presence of 25 μg/ml. chloramphenicol observed in the experiment of Fig. 2 shows that the evolution of decay resistance of the infective center is a rather sensitive indicator of the *residual* protein synthesis which still proceeds at 5% of its normal rate at this concentration of antibiotic (Hershey, 1956; Aronson & Spiegelman, 1958). Since previously published experiments on the relation of protein synthesis to DNA synthesis in phage-infected bacteria employed concentrations of chloramphenicol no higher than 30 μg/ml. (Tomizawa & Sunakawa, 1956; Hershey & Melechen, 1957; Crawford, 1957), we have now examined this relation at higher antibiotic concentrations, which reduce the rate of protein synthesis to less than 1% its normal value (Hershey, 1956; Aronson & Spiegelman, 1958).

(i) *Phage T1*

An experiment was carried out to study the effect of 300 μg/ml. chloramphenicol on DNA synthesis in T1-infected bacteria. For this purpose, the amount of ^{32}P was measured which enters the DNA fraction of uninfected and T1-infected bacteria in the presence and absence of the antibiotic. The results of this experiment are presented in Fig. 3, where the quantity of newly synthesized DNA is expressed in terms of *T1 phage* units per cell. It can be seen in Fig. 3 that the amount of phosphorus which enters the DNA fraction in the absence of chloramphenicol is at first very nearly the same in T1-infected and in uninfected cultures, although the lysis of the infected cells finally puts an end to further DNA synthesis in the infected culture. The results of

Fig. 3 show, furthermore, that also in the presence of 300 μg/ml. chloramphenicol similar amounts of newly assimilated phosphorus enter the DNA fraction in T1-infected and uninfected cultures. The kinetics of DNA synthesis in the presence of the antibiotic are, however, rather different from those in its absence, in that in the presence of chloramphenicol assimilation of new phosphorus into the DNA fraction

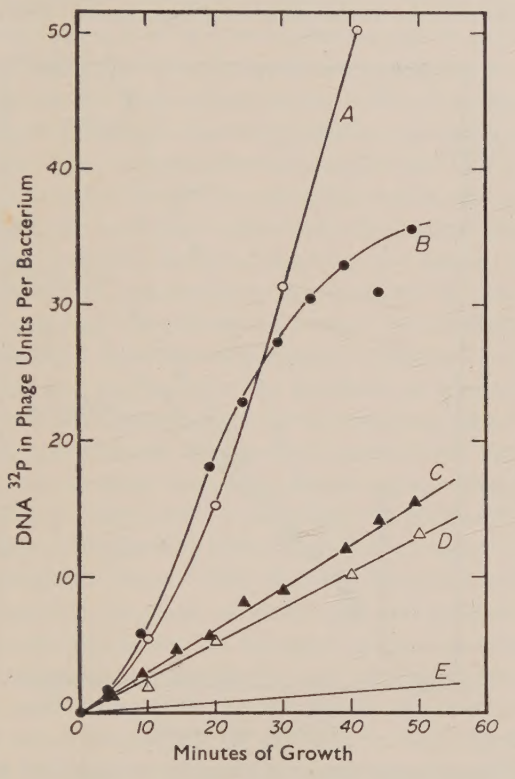

Fig. 3. DNA synthesis in uninfected bacteria (curves A and D) and in T1-infected bacteria (curves B and C) in the absence (curves A and B) and presence (curves C and D) of 300 μg/ml. chloramphenicol. Curve E: estimated maximum contribution of uninfected bacteria to curve C.

Procedure. A standard suspension of R2 bacteria was centrifuged and resuspended in T1 adsorption medium, treated with chloramphenicol and finally brought into 0·01 M-KCN, exactly as in the experiment described in Fig. 2. One portion of this culture was then infected with a multiplicity of 4 T1 phages/cell, and phage adsorption allowed to proceed for 8 min. At this time, the infected culture and another portion of the uninfected culture were centrifuged, washed once, and resuspended in tris-buffer. Infected and uninfected cultures were then each subdivided into two parts. To one of the infected and to one of the uninfected cultures chloramphenicol was added to a final concentration of 300 μg/ml. Growth was then initiated in all 4 tubes simultaneously by addition of glucose and casamino acids. At the same time, ^{32}P at a specific activity of 0·64 mC/mg P was also added to each tube. Plaque and colony assays made 2 min later showed that there were $3{\cdot}1 \times 10^8$ infective centers/ml. and $6{\cdot}0 \times 10^7$ surviving uninfected bacteria/ml. in the two infected cultures and $4{\cdot}1 \times 10^8$ viable cells/ml. in the two uninfected cultures. From time to time, samples from each of the cultures were diluted into ice-cold M-trichloroacetic acid. The samples were then fractionated chemically and the amount of ^{32}P in the DNA fraction of each counted.

appears to proceed at a constant rather than increasing rate, equal to the initial rate of assimilation in the two cultures without chloramphenicol. The maximum contribution of any *uninfected* bacteria to the total DNA synthesis observed in the T1-infected, chloramphenicol-inhibited culture has been estimated, and, as can be seen in Fig. 3, is quite negligible. Hence it may be inferred that bacteria infected with T1 phage in the presence of 300 μg/ml. chloramphenicol do continue to synthesize DNA at the same

rate at which they were synthesizing DNA just prior to infection, even though under these same conditions the infecting T1 phage cannot attain the decay-resistant state.

(ii) *Phage T2*

Figure 4 presents the result of a study of DNA synthesis in T2-infected bacteria to which 100, 400 or 800 μg/ml. chloramphenicol has been added at the 11th minute of intracellular phage growth. As in the preceding experiment, DNA synthesis has been

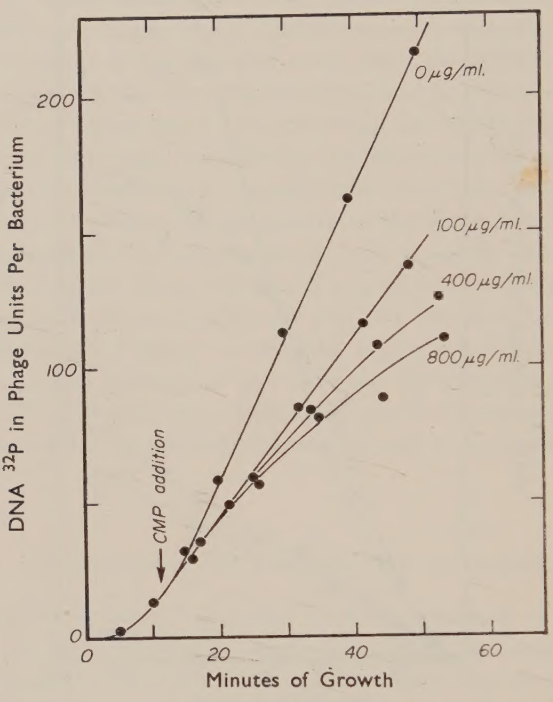

FIG. 4. The effect of various concentrations of chloramphenicol in DNA synthesis in T2-infected bacteria.

Procedure. A standard suspension of R2 bacteria was infected at a multiplicity of 4 phages/cell with a purified stock of T2. After allowing phage adsorption to proceed for 5 min, the infected bacteria were sedimented to remove unadsorbed free phage and resuspended in tris buffer. Plaque and colony assays made at this point indicated $2{\cdot}5 \times 10^8$ infective centers/ml. and $2{\cdot}1 \times 10^7$ surviving uninfected bacteria/ml. in the culture. Phage growth was then initiated by addition of glucose and casamino acids to the infected bacterial suspension. At the same time, ^{32}P was added to the culture to a specific activity of 0·43 mc/mg P. Eleven min later the culture was subdivided into 4 portions to which chloramphenicol was added to final concentrations of 0, 100, 400, and 800 μg/ml. respectively. From time to time, samples of each of the growth tubes were diluted into ice-cold 0·3 M-trichloroacetic acid. These samples were then fractionated and the amount of ^{32}P in the DNA fraction of each counted.

estimated here by the uptake of ^{32}P into the DNA fraction of the infected culture. It is evident that DNA synthesis does proceed in T2-infected cells to which chloramphenicol concentrations as high as 800 μg/ml. have been added after formation of the "early" protein, although the final rate of DNA synthesis is somewhat lower the higher the concentration of antibiotic added.

4. Discussion

The mechanism underlying the gradual ^{32}P-decay stabilization at the outset of vegetative growth of ^{32}P-labeled phages is not yet understood, although some more or less plausible explanations have been advanced (Stent, 1955, 1958; Symonds &

McCloy, 1958; Stahl, 1959). One of these theories envisages that the infective center becomes refractory to decay inactivation as replicas of the parental DNA make their intracellular appearance. Once such replicas are present, mature phage progeny can issue from the infected cell, even after lethal ^{32}P disintegrations have destroyed the labeled parental DNA molecules. An infective center in which some degree of radiostabilization intermediate between full sensitivity and complete refractoriness exists would, from this point of view, represent a bacterium in which viral DNA replication has already commenced but not yet proceeded far enough to produce the final stabilization. The present finding that protein synthesis is required not only for the onset of the stabilization process but throughout its course would be incompatible with this hypothesis, if it is assumed that the necessary protein represents only the enzymes of phage DNA metabolism. For, once stabilization (and hence DNA replication) is under way, the new enzymes required for DNA replication would, *nolens volens*, already be present, and DNA synthesis (and hence stabilization) should continue in the absence of any further protein synthesis. In fact, whatever the nature of the stabilization reaction, it seems difficult to imagine how the synthesis of new enzymes of DNA metabolism could be required throughout its course. The need for protein synthesis also during the intracellular stabilization of phage T1 renders it even less likely that it is the appearance of new enzymes for the synthesis of phage-specific DNA components which limits the attainment of the decay-resistant state.

If the stabilization phenomenon does indeed derive from the replication of the infecting viral DNA, then it would seem necessary to assume that the synthesis of the "early" protein gradually "activates" a progressively greater part of the parental DNA, allowing the onset of replication of the already "activated" parts. For instance, the idea proposed previously (Stent, 1958) that DNA replication proceeds indirectly through a ribonucleoprotein template, to which the viral genetic information is first transferred, would fit with this notion. At the stage at which stabilization has attained a level intermediate between full sensitivity and complete refractoriness only disintegrations in that part of the DNA whose genetic information has not yet been "transferred" to the ribonucleoprotein should then be lethal to the infective center. Inhibition of protein synthesis at this stage would arrest any further stabilization because transfer of the remaining information to ribonucleoprotein has been stopped. An alternative idea, involving no information transfer, would be that some newly synthesized protein has to combine with the infecting viral DNA prior to the replication process. This hypothetical "activator" protein could be the *internal* protein of the T-even phages, which constitutes about 5% of the protein of the mature virus, is injected into the infected cell together with the parental DNA, and is then synthesized at the very start of intracellular phage growth before the onset of DNA replication (Levine, Barlow & Van Vunakis, 1958; Murakami, Van Vunakis & Levine, 1959). Under this idea an infective center of intermediate decay sensitivity represents a bacterium in which some parts of the parental DNA complement have already received their internal protein and commenced replication while others have not. Thus only ^{32}P disintegrations occurring in the as yet unduplicated fraction of the parental DNA could abort the ultimate appearance of infective progeny. Here, inhibition of protein synthesis prevents the further progress of stabilization because no more labeled parental DNA can be "activated" to commence its replication.

Finally, one could entertain the notion that decay stabilization of the parental phage DNA does not, after all, derive from its replication, or genotypic expression, but that, instead, the synthesis of virus-related enzymes and structural proteins

induced by the viral genome, or *phenotypic* expression of the DNA, is responsible for gradually rendering the infective center refractory to decay. From this point of view, an infective center of intermediate decay sensitivity represents a bacterium in which part of the parental DNA molecules have already induced the synthesis of enough of their gene products so that further phenotypic function of that part of the DNA molecules can be destroyed by lethal ^{32}P disintegrations without suppressing the ultimate appearance of infective progeny. The residual ^{32}P decay sensitivity of such an infective center then reflects that other part of the parental DNA which has not yet given sufficient phenotypic expression and whose radiochemical destruction at this stage aborts successful virus reproduction. Inhibition of protein synthesis here arrests further stabilization because it prevents the appearance of gene products by the still decay-sensitive fraction of the parental DNA. This last explanation of the stabilization process, however, would have different consequences for the function of the viral genome depending on whether replication of the various parts of the parental DNA followed or preceded their phenotypic expression. If phenotypic expression preceded replication, then the postulated decay refractory state of the phenotypically expressed DNA molecules would demand that the *genetic survival* of the phage DNA (i.e., its ability to induce the synthesis of replicas of itself) is much less decay-sensitive than its *functional* survival (*cf.* Symonds & McCloy, 1958). This actually appears to be the case for the DNA of the bacterial nucleus, where the results of conjugation experiments demonstrate the genetic survival of gene loci whose phenotypic expression has already been abolished by ^{32}P decay (Fuerst, Jacob & Wollman, 1956; McFall, Pardee & Stent, 1958; Riley, Pardee, Monod & Jacob, 1960). If phenotypic expression followed replication, on the other hand, then the decay sensitivity of the non-expressed but already replicated DNA molecules would require that only the parental DNA molecules, but not their replicas, are competent to induce phenotypic expression in the infected cell. This appears to be true for the function of the r_{II} region of phage T4, where the appearance of *de novo* r_{II}^{+} revertants or recombinants during the abortive growth of r_{II} mutants in the differential host K12(λ) cannot save the infective center (Krieg, 1959; Pratt & Stent, 1959).

The finding that in the presence of chloramphenicol T1-infected bacteria do synthesize DNA without attaining the decay-refractory state may now be interpreted in the following way. If stabilization of the infecting phage does derive from the replication of its DNA, then it can be concluded that the post-infection DNA synthesized in chloramphenicol-inhibited T1 infective centers represents simply the continuation of *bacterial* DNA anabolism, and not the formation of viral DNA. On the other hand, if phenotypic expression of the parental DNA is responsible for the stabilization process, then it is possible that the post-infection DNA made in the presence of the antibotic is actually T1 DNA, whose replication can commence without synthesis of any "early" protein.

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Protein Synthesis and the Onset of Intracellular Bacteriophage Growth

II. Stabilization to ^{32}P Decay of the Genome of a Secondary Phage †

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Experiments have been carried out to test for the "individually specific" nature of the protein which T2 bacteriophages synthesize at the outset of their intracellular growth and which is necessary for attaining the ^{32}P decay refractory state of the vegetative phage. For this purpose, bacteria were infected with non-labeled T2$h^+r_7r_1^+m^+$ and intracellular growth allowed to proceed for 11 min before protein synthesis was arrested by addition to the culture of 300 μg/ml. chloramphenicol. At this time, the already infected bacteria were *superinfected* with a highly ^{32}P-labeled stock of the complementary T2$hr_7^+r_1m$ genotype and the culture incubated further in the presence of the antibiotic. Portions of the culture were frozen and stored after various additional incubation periods and the survival in the superinfected complexes of the three distantly linked secondary loci h, r_7^+ and r_1 followed as a function of radioactive decay. It was found in this way that the genetic markers of the secondary parent do not become refractory to decay inactivation during incubation of the superinfected complexes in chloramphenicol. Hence the protein synthesized during the initial growth of the primary phage cannot serve for the subsequent radiostabilization of the secondary phage, which must be allowed to induce the formation of its own protein in order to attain the decay refractory state. Some component of the "early" protein must, therefore, be "individually specific."

1. Introduction

It was shown in the preceding paper of this series (Uchida & Stent, 1960) that the synthesis of protein is necessary throughout the stabilization reaction responsible for the evolution of the decay-refractory state of highly ^{32}P-labeled bacteriophages at early stages of their intra-cellular growth. The experiments to be described now were designed to elucidate whether this protein is *individually specific*, i.e., whether in a bacterium infected with more than one virus *every* viral genome must induce the synthesis of its own protein before it can attain the ^{32}P decay refractory state, or whether this protein is non-specific, in the sense that the protein synthesized by one virus can also serve for the radiostabilization of other labeled phages within the same cell. The latter alternative should obtain if this essential protein is a system of metabolic enzymes which modifies the general physiological conditions of the infected bacterium.

The plan of the present experiments is to infect bacteria primarily with a population of non-radioactive T2 phages, allow normal intracellular phage growth until the "early" protein has been formed and the infected cells are ready to synthesise phage-

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specific DNA at the full rate. At this time, further protein synthesis is arrested by addition of chloramphenicol. The infected cells are then *superinfected* with secondary, highly ^{32}P-labeled T2 phages, which differ from the primary virus in several genetic markers. The secondarily infected culture is incubated further in the presence of the antibiotic and portions frozen from time to time and stored at — 196°C in order to examine the ^{32}P decay sensitivity of the genetic markers introduced into the infected cell by the secondary phage. Now if, on the one hand, the protein synthesized under the influence of the primary phage prior to the addition of the antibiotic is not individually specific, then in this experiment the protein of the primary phage ought to serve also for the radiostabilization of the genetic markers of the secondary phage, which entered the cell only after protein synthesis had been arrested. If, on the other hand, the essential protein *is* individually specific, then the protein of the primary phage cannot serve for the stabilization reactions of the secondary phage and the secondary, radioactive genetic markers ought to remain sensitive to inactivation by decay as long as the secondary phage has had no opportunity to synthesize its own essential protein.

2. Materials and Methods

Except as noted below, the bacterial strains, methods of phage growth, composition of media, and radiochemical techniques were the same as those described in the preceding paper (Uchida & Stent, 1960).

Phage strains. The mutants r_7 and hr_1m of bacteriophage T2(H) (Hershey & Rotman, 1949).

Bacterial strains. The following strains of *Escherichia coli* were used as indicators for plaque assays: Strain BB (McFall & Stent, 1958); strain B/4/2, a mutant of Hershey's strain S resistant to T4 and T2; and strain K12S(λ) (referred to here as K), an artificial lysogenic strain derived from λ-sensitive strain of K12 of Lederberg & Lederberg (1953). Cultures of these indicator bacteria were grown overnight with aeration at 30°C in casamino acid glycerol medium.

Plaque assays. Phage assays were made on tryptone broth agar plates seeded with either strain BB (on which the r_7 mutant shows the r^+ phenotype), or with mixed indicators composed of one part B/4/2 to ten parts of K. Only those infective centers produce clear plaques on such mixed indicators which yield both h and r_7^+ alleles, since the h^+ wild type cannot grow on strain B/4/2 and the r_7 mutant cannot grow in strain K. The plaques produced on mixed indicators were scored after incubation of the assay plates at 37°C for 20 to 24 hr.

3. Experimental

(a) *Rescue of genetic loci after ^{32}P decay*

In the experiment described in the Introduction, the kinetics of decay inactivation of the genetic markers of the secondary, radioactive phage are evidently complicated by the existence of *cross-reactivation*, or *rescue* of these markers, by the fully viable, non-radioactive, complementary genome of the primary phage already present in the same cell. Let us consider an infected cell in which decay is allowed to proceed immediately after the adsorption of the ^{32}P-labeled secondary virus particles, i.e., before their stabilization can have occurred. It is not to be expected that even in such a cell the inactivation of the secondary markers will proceed at the rate of death of the extracellular free labeled phage, since only a minor fraction of the lethal ^{32}P disintegrations destroys the ability of a given genetic locus to participate in the reproductive events initiated in the same cell by a homologous, viable phage (Stent, 1953; Stahl,

1956). In order to determine, therefore, the extent of marker rescue to be anticipated in this experiment, a cross-reactivation study was first undertaken with the phage strains intended here for use as primary and secondary phages.

For this purpose, bacteria were infected with highly ^{32}P-labeled T2 phage bearing the four distantly linked loci $hr_7^+r_1m$ (Hershey & Rotman, 1949) and infected shortly thereafter with non-labeled phages of the complementary genotype $h^+r_7r_1^+m^+$. The mixedly infected bacteria were then stored at — 196°C, so that the survival of the genetic markers of the labeled phage with radioactive decay could be followed by plating the frozen infective centers directly on appropriate indicator bacteria from time to time. Only those h, r_7^+ and r_1 markers of the labeled phage were scored which appeared in plaques also containing the m^+ marker of the non-labeled parent, in order to follow marker survival exclusively in infective centers infected with *both* labeled and non-labeled genotypes. The results of this experiment are presented in Fig. 1. It can be seen there that radioactive decay rapidly inactivates the free labeled T2$hr_7^+r_1m$, as well as the infective centers in which only the labeled parent is present (curves A and B). On the other hand, it is evident that the number of mixedly infected bacteria yielding both the r_1 marker of the labeled parent and the m^+ marker of the non-labeled parent decays much more slowly (curve D). This indicates that after lethal ^{32}P disintegrations have destroyed the reproductive potential of the labeled parent, its r_1 marker can still be rescued efficiently in mixed infection with a viable, non-labeled phage genome. The inactivation of the r_1 locus is seen to proceed according to a concave curve, whose initial and final slopes are only 0·1 and 0·02 respectively of the rate of inactivation of the free virus. The *simultaneous* survival of the three markers h, r_7^+, and r_1 of the labeled parent in mixed infection with a non-labeled complementary genotype contributing its m^+ marker is seen to decay more rapidly than the survival of the r_1 locus alone (curve C). Inactivation of infective centers yielding the three markers of the labeled parent also proceeds according to a concave curve, whose initial and final slopes are approximately three times greater than the corresponding slopes of the single r_1 marker survival curve.

These marker survival curves are qualitatively similar to those observed by Doermann & Chase (*cf.* Stahl, 1959) for cross-reactivation of genetic loci in T4 phage after ultraviolet light inactivation, and the nature of the ^{32}P decay marker inactivation kinetics can be explained in a manner formally analogous to that by which Doermann & Chase accounted for their observations. One may envisage that the crossreactivation process rescues markers of ^{32}P-decay inactivated phage by a mechanism of genetic exchange; hence a marker which has been "knocked out" is one in which a lethal disintegration has either destroyed the marker itself or prevented the exchange process. The initial and final slopes of the concave r_1 marker survival curve, and the intercept of 0·4 of the extrapolation of its final asymptote to zero decay, then imply that the chance that a lethal ^{32}P disintegration knocks out the r_1 locus varies from 0·1 to 0·02 in different members of the labeled phage population, the lower knockout efficiency obtaining in approximately 0·4 of the individuals. This variability in the sensitivity of one and the same marker in different virus particles need not necessarily reflect any *a priori* heterogeneity of the parental phage population, but could represent some chance element inherent in the genetic exchange process which rescues markers from the moribund genome. (Similarly concave survival curves are observed in the ^{32}P decay inactivation of genetic loci of labeled bacterial chromosomes in conjugational merozygotes. Fuerst, Jacob & Wollman; *cf.* Stent & Fuerst, 1960.) The most likely interpretation of the minimum knockout

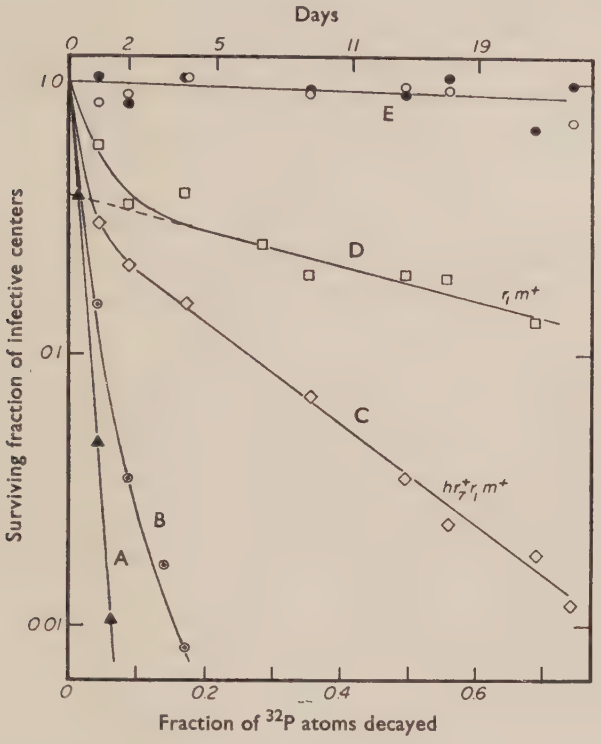

FIG. 1

Rescue of genetic markers of ^{32}P-labeled T2. Curve A: survival of the labeled T2$hr_7^+ r_1 m$ parent. Curve B: survival of T2$hr_7^+ r_1 m$ infective centers after phage development for 0·5 min. Curves C and D: survival of the ability to yield the markers $hr_7^+ r_1 m^+$(C) or $r_1 m^+$ (D) of infective centers mixedly infected with labeled T2$hr_7^+ r_1 m$ and non-labeled T2$h^+ r_7 r_1^+ m^+$. Curve E: survival of the ability to yield the markers $r_1 m$ (open circles) or $hr_7^+ r_1 m^+$ (closed circles) of infective centers primarily infected with labeled T2$hr_7^+ r_1 m$ and secondarily infected with T2$h^+ r_7 m^+$ 11 min later.

Procedure. A standard suspension of R2 bacteria was infected with $1{\cdot}2 \times 10^8$ viable phage particles per ml. of a nutrient-free radioactive stock of T2$hr_7^+ r_1 m$ labeled with ^{32}P at a specific activity of 820 mc/mg P and phage adsorption allowed to proceed for 5 min; colony survival counts of the infected culture at this time indicate an average multiplicity of infection of 0·31 phages/cell. The infected culture was sedimented and resuspended in 0·5 ml. tris-buffer, to which glucose and casamino acids were added at $t = 0$ to start intracellular phage growth. At $t = 0{\cdot}5$ min, a portion of the culture was cooled and numerous samples frozen and stored. At the same time, 300 μg/ml. chloramphenicol was added to a second portion of the culture and the bacteria superinfected with a multiplicity of about 1·5 non-labeled T2$h^+ r_7 r_1^+ m^+$ phages per cell. At $t = 11$ min a third portion of the infected culture was similarly super-infected with unlabeled T2$h^+ r_7 r_1^+ m^+$ in the presence of the antibiotic. The two superinfected cultures were incubated until $t = 14$ min, when they were cooled and numerous samples of them, as well as of the radioactive T2$hr_7^+ r_1 m$ parental phage stock, frozen and stored. From day to day, one sample of each frozen set was thawed and plated immediately on agar seeded either with strain BB, or with the mixed indicators K + B/4/2, for assay of the surviving number of infective centers. On the plates seeded with BB, any mixedly infected bacterium will give rise to a large (i.e., non-minute $m+$) r or mottled type plaque if at least one copy of the r_1 allele of the radioactive parent and one copy of the $m+$ allele of the non-radioactive parent appears among the progeny. On the plates seeded with the mixed indicators, any mixedly infected bacterium will give rise to a clear, large r or mottled type plaque if it yields at least one progeny particle of genotype $hr_7^+ r_1 m^+$, i.e. one carrying three markers of the labeled parent and one marker of the unlabeled parent. (Though sufficient, it may not actually be necessary for the formation of this plaque type that the 4 markers in question appear within the *same* progeny particle. For instance, an infected cell yielding among its progeny the genotypes $h^+ r_7^+ r_1 m^+$ and $hr_7^+ r_1^+ m^+$ would probably also produce the same plaque type.)

efficiency of 0·02 is that any lethal ^{32}P disintegration occurring within a polynucleotide envelope around the marker amounting to about 2% of the DNA of the T2 phage particle (or to a molecular weight of 2×10^6) is certain to prevent rescue of the marker. The threefold greater decay sensitivity of the simultaneous survival of the three distantly linked loci h, r_7^+, and r_1 indicates that the rescue of each of these markers from a moribund genome is an event independent of the rescue of the other two markers.

It should be noted that the cross-reactivability of the r_1 marker observed here is very much greater than that previously reported (Stent, 1953). For in our earlier experiments it was found that radioactive decay inactivates the r_1 locus in a more or less simple exponential manner at about 0·4 the rate of inactivation of the entire phage. The discrepancy between the two sets of results probably arises from the fact that in the earlier experiments labeled and unlabeled phages were adsorbed to bacteria prior to decay in the presence of 0·01 M-KCN, whereas no cyanide was present in the present procedure. It seems, therefore, that some early step in intracellular phage development must proceed prior to decay in the DNA of the labeled parent phage before efficient cross-reactivation can occur. This step is evidently inhibited by KCN, but, as will be evident from the data presented later in Fig. 2, is not inhibited by chloramphenicol. The genetic effects of ^{32}P disintegrations thus resemble those of X-rays, where an "early step damage" (Weigle & Bertani, 1956) likewise greatly reduces the cross-reactivation efficiency when phages are irradiated in either the free state or after adsorption to bacteria in the presence of KCN, compared to the highly efficient marker rescue observable after irradiation of bacteria infected in the presence of chloramphenicol (Harm, 1958).

(b) *Effect of chloramphenicol on the stabilization of the secondary phage genome*

We are now ready to consider the experiment designed to test whether the "early protein" plays an individually specific role in intracellular phage growth. Since, as was seen in Fig. 1, cross-reactivation of a single locus is so highly efficient in this system that elimination of individual markers proceeds at rather slow rate, it is necessary to make use of the much greater sensitivity of the *simultaneous* survival of the three distantly linked loci h, r_7^+, and r_1 of the secondary phage. For it would be difficult to notice any effect of protein synthesis on the radiostabilization of the secondary genome if already at the outset the rate of inactivation of the markers followed for this purpose is very low. Accordingly, bacteria were primarily infected with non-labeled T2$h^+r_7r_1^+m^+$ particles and, after synthesis of the "early protein" had been allowed for 11 min, secondarily infected with highly ^{32}P-labeled T2$hr_7^+r_1m$ particles. In this way, the sensitivity to radioactive decay of the joint survival of h, r_7^+ and r_1 loci could be followed after various incubation periods. These three markers of the secondary phage were always scored in association with the m^+ marker of the primary phage, in order to make certain that only the phage progeny of bacteria infected with *both* primary *and* secondary phages entered into consideration.

The outcome of this experiment is shown in Table 1 and Fig. 2. Table 1 presents the results of assays prior to radioactive decay. It is apparent, first of all, that by the 11th minute after onset of phage growth only 1·5 to 2·1% of the primarily infected bacteria can be superinfected to yield the three distantly linked loci h, r_7^+, and r_1 of the secondary parent. (The secondary marker r_1 can be seen to appear in about 4% of the primarily infected bacteria.) This exclusion of secondary phages from primarily infected bacteria appears to be connected with the superinfection breakdown mech-

anism initiated by the intracellular growth of T-even phages (Dulbecco, 1952; Graham, 1953). However, if a very high multiplicity of several hundred secondary phages per primarily infected cell is employed, then the secondary genetic loci can be successfully introduced into the vegetative phage pool of most of the primarily infected cells (Visconti & Garen, 1953). Such extremely high multiplicities of secondary infection could not be employed in the present experiments, because it is not

TABLE 1

Efficiency of the superinfection process and stability of the infective centers in chloramphenicol

Sample	Assays on BB			Assays on K + B/2/4	
	Infective centers	Infective centers yielding $r_1 m^+$		Infective centers yielding $hr^+_7 r_1 m^+$	
	Titer	Titer	% of total	Titer	% of total
Primary infection only	†$1{\cdot}25\times10^8$				
Secondary infection in chloramphenicol					
$t = 17$ min	$1{\cdot}12\times10^8$	$4{\cdot}5\times10^6$	4·0	$1{\cdot}7\times10^6$	1·5
$t = 34$ min	$1{\cdot}12\times10^8$	$5{\cdot}2\times10^6$	4·6	$2{\cdot}0\times10^6$	1·8
$t = 44$ min	$1{\cdot}15\times10^8$	$3{\cdot}0\times10^6$	2·7	$2{\cdot}4\times10^6$	2·1
Secondary infection without chloramphenicol					
$t = 21$ min	$1{\cdot}73\times10^8$‡	$4{\cdot}5\times10^6$	(4·0)§	$2{\cdot}5\times10^6$	(2·2)§

†Assay before freezing ($t = 2$ min).
‡Includes some progeny phage.
§Based on total titer of $1{\cdot}12\times10^8$.

feasible to prepare very high titer stocks of highly ^{32}P-labeled phages. Secondly, it is evident from Table 1 that incubation of the infected bacteria in 300 μg/ml. chloramphenicol does not seriously diminish the total titer of infective centers and does not radically change the proportion that yield the three secondary markers. The assays reported in Table 1 were carried out on samples which had been frozen and thawed. Analogous assays before freezing showed that more than 85% of the infective centers, both total and secondarily infected, survive the freezing and thawing process.

Figure 2 presents the survival of the secondarily infected bacteria producing large, clear *r*-type plaques on the mixed indicators K + B/4/2 (i.e., liberating the loci *h*, r^+_7, r_1, and m^+) as a function of the fraction of ^{32}P atoms decayed. These results show that in the sample superinfected in the presence of 300 μg/ml. chloramphenicol and incubated in the presence of the antibiotic for 6 min, subsequent radioactive decay destroys the joint appearance of the three secondary markers in a manner identical to the inactivation of the same three markers in the cross-reactivation experiment presented in Fig. 1. Hence it may be concluded that the mechanism of marker rescue is the same whether the cross-reactivating non-labeled phage present in the bacterium has or has not yet started its intracellular development prior to decay inactivation of the labeled genome. It can be seen, furthermore, that decay similarly suppresses

the joint appearance of the three markers of the secondary phage in the two samples incubated until the 34th and the 44th min in the presence of the antibiotic. Chloramphenicol, therefore, does appear to prevent the stabilization to decay inactivation of the labeled secondary genome in the superinfected cells. It should be noted, however, that the inactivation kinetics of these later samples are not completely identical with those of the sample frozen at the 17th min, in that the final slope of the three marker

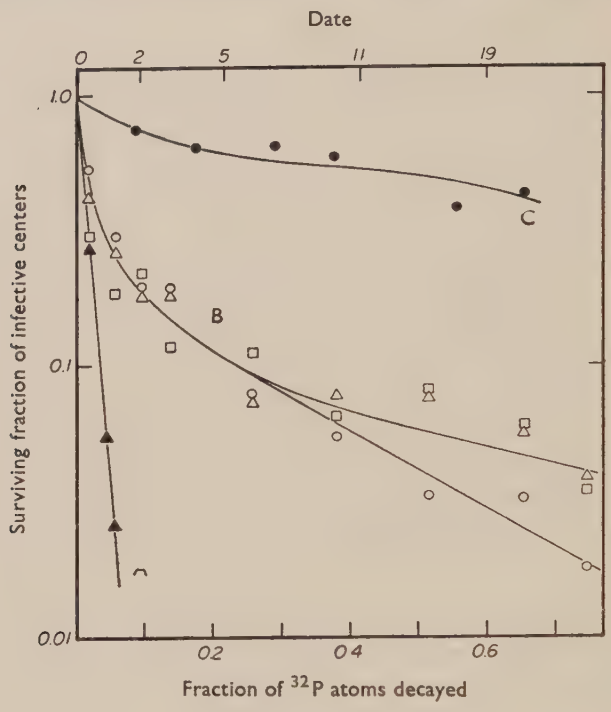

FIG. 2

The effect of 300 μg ml. chloramphenicol on the decay sensitivity of the genome of ^{32}P-labeled secondary phages infecting bacteria in which intracellular growth of a non-labeled primary phage is already under way. Curve A: survival of the labeled T2$hr^{+}_{7}r_{1}m$ parent. Curves B and C: survival of the ability to yield the markers $hr^{+}_{7}r_{1}m^{+}$ of infective centers primarily infected with non-labeled T2$h^{+}r_{7}r^{+}_{1}m^{+}$ and secondarily infected with labeled T2$hr^{+}_{7}r_{1}m$. Curve B: secondary infection at the 11th and further incubation in the *presence* of chloramphenicol until the 17th (circles), 34th (triangles) and 44th (squares) minute of phage growth. Curve C: secondary infection at the 12th and further incubation in the *absence* of chloramphenicol until the 21st minute of phage growth.

Procedure. A standard suspension of R2 bacteria was infected with a purified stock of T2$h^{+}r_{7}r^{+}_{1}m^{+}$. Adsorption was allowed to proceed for 5 min, at which time colony survival counts of the infected culture indicated an average multiplicity of infection of 1·7 phage particles per cell. The infected culture was sedimented, washed once to remove any free unadsorbed phages, and finally resuspended in tris-buffer to which glucose and casamino acids were added at $t = 0$ to start intracellular phage growth. At $t = 11$ min, chloramphenicol was added to a portion of the infected culture to a final concentration of 300 μg/ml. and the portion superinfected with a multiplicity of 1·1 T2 $hr^{+}_{7}r_{1}m$ phages per cell, labeled at a specific ^{32}P activity of 820 mc/mg P. At $t = 12$ min, a second portion of the original infected culture was similarly infected secondarily with the labeled T2$hr^{+}_{7}r_{1}m$ but in the absence of chloramphenicol. At $t = 14$ min, the chloramphenicol-inhibited culture was diluted 17·5-fold in tris-glucose medium containing anti-T2 serum ($k = 6$ per min) and 300 μg/ml. chloramphenicol. At $t = 15$ min, the other culture, containing no chloramphenicol, was similarly diluted, except that here the dilution medium contained no chloramphenicol. At various times, portions of the two infected cultures were cooled and numerous samples of each portion, as well as of the free radioactive T2$hr^{+}_{7}r_{1}m$ parental phage stock, were frozen and stored. From day to day, one sample of each frozen set was thawed and plated immediately on agar seeded with strain BB or with the mixed indicators K + B/4/2.

survival curve in the later samples appears to be somewhat reduced. Finally, it is evident from curve C of Fig. 2 that a considerable stabilization to inactivation by decay has occurred in a control sample in which the secondary infection was carried out in the absence of chloramphenicol and normal intracellular growth was allowed to proceed for 9 min prior to freezing and storage. This stabilization is not complete, however, in that the fraction of infective centers liberating all three secondary markers decreases with decay at a low but significant rate. It is unfortunately not possible to examine whether longer incubation in the absence of the antibiotic does produce a complete stabilization of the radioactive secondary markers, since the approaching end of the latent period renders freezing and storage of the infective centers impossible after the primary phage has already grown for more than 21 min within the bacteria. The results of the day-to-day assays on agar seeded with strain BB are not presented in Fig. 2. These assays merely showed that the *total* number of infective centers recoverable from the frozen samples did not decrease with radioactive decay, i.e., that the successful course of the primary infection initiated by the unlabeled phage is stable to the conditions of storage.

This experiment thus indicates that the "early" protein appears to play an individually specific role, in that the protein induced by the non-labeled primary phage cannot serve for the radiostabilization of the genetic loci of the ^{32}P-labeled secondary phage. On the contrary, the secondary phage genome must have an opportunity to induce the synthesis of its own "early" protein in order to achieve the ^{32}P decay resistant state.

(c) *Development of primary phage in superinfectable bacteria*

An important control is, however, necessary before the conclusion just stated can actually be accepted. As was evident in Table 1, only a minor proportion of the primarily infected cells can be successfully superinfected 11 min after the onset of phage growth to yield the loci of the secondary phage. Since it is known that the exclusion of the secondary phage becomes progressively more pronounced the further the primary phage has been allowed to develop (Dulbecco, 1952), it is conceivable that the superinfectable bacteria represent a special fraction in which the growth of primary phage has progressed more slowly than in the majority of the population. It is possible, therefore, that the "early" protein of the primary phage has not as yet been made in these secondarily infected cells, even though the protein is already present in that majority of primarily infected bacteria from which the secondary genome might be excluded. If this is actually the case, then the genome of the secondary phage could not wax refractory to decay inactivation in the absence of protein synthesis, whether the "early" protein is individually specific or not. Hence it is necessary to show that at the time of addition of antibiotic and secondary infection, the "early" protein has already been made not only in the majority of primarily infected cells (which is attested to by the rapid, subsequent, global synthesis of phage DNA, as seen in Fig. 4 of the preceding paper) but also in that minority of the bacteria into which the secondary phage markers can be successfully introduced. It is possible to test this point by reversing the role of the two phage stocks used in the experiment of Fig. 2, i.e., by making the primary infection with the highly ^{32}P-labeled T2$hr^{+}_{7}r_{1}m$ phages and the secondary infection 11 min later with the non-labeled T2$h^{+}r_{7}r^{+}_{1}m^{+}$ phages in the presence of chloramphenicol. Now, by examining the effect of radioactive decay on the genetic loci $hr^{+}_{7}r_{1}$ of the primary phage in those infective centers which also yield the m^{+} locus of the secondary phage one can

determine whether the genetic markers of the primary phage have, in fact, become stabilized against decay inactivation in that minority of the bacteria into which markers of the secondary phage can be introduced.

The results of this control experiment are presented as curve E in Fig. 1. It is seen here that neither the infective centers infected only with the primary phage (i.e., that majority of the population which does not yield the m^+ marker) nor the infective centers infected with *both* primary *and* secondary phage (i.e., that 2·5% of the population which yields the markers $hr_7^+r_1m^+$) are inactivated appreciably by radioactive decay. It may be concluded, therefore, that during the first 11 min of intracellular phage growth the primary phage has already completed those reactions which lead to stabilization, not only in the majority of the population of infected cells, but also in that minority which still can be superinfected to yield genetic loci of the secondary phage. Consequently the protein necessary for the radiostabilization of the primary genome must have already been produced in the superinfectable cells. Thus our earlier inference derived from the data of Fig. 2 that the labeled secondary phage genome needs to synthesize its own protein to attain the decay-resistant state, even though the protein induced by the primary phage is already present in the same cell, appears justified.

4. Discussion

The present finding that the protein synthesized in the course of the stabilization reaction contains an "individually specific" component lends further support for the argument advanced in the first paper of this series that the enzymes of metabolism of phage-specific DNA components cannot be the only "early" proteins involved in this process. The other hypotheses which we considered previously concerning the nature of this protein and its relation to the stabilization reaction can, however, still be reconciled with the present result. In fact, if the stabilization phenomenon does derive from the replication of the infecting viral DNA, then the "activator" function which we had assigned to the "early" protein would actually demand that its role be "individually" specific. In particular, the ribonucleoprotein, as hypothetical intermediate template of DNA replication, would naturally serve only for the replication of those DNA molecules whose information had been transferred to it, while the *internal* protein, as postulated activator of replication, would necessarily activate the replication of only those DNA molecules to which it is already attached. In either case, the genetic markers of the ^{32}P-labeled secondary phage could not become stabilized until the secondary DNA had induced the synthesis of its own ribonucleoprotein or internal protein.

The situation is more complicated, however, if stabilization derives not from replication of the infecting parental DNA but rather from its phenotypic expression. For in the superinfection experiments carried out here it might have been thought that the non-labeled viable primary genome, always present together with the labeled secondary genome, is fully competent to supply all the gene products required for the construction of infective progeny. Indeed, the rather low probability of single marker knockout of 0·1 to 0·02 per lethal ^{32}P disintegration shows that the viable partner can provide many of the physiological functions to the ^{32}P-decay damaged labeled genome. There seems to be no *a priori* reason, however, why even the low marker knockout probability should not represent the ultimate destruction of some physiological function which the DNA molecule bearing the marker in question must perform for itself before the marker, or any of its replicas, can appear in infective progeny through

the cross-reactivation process. Hence the genetic markers of the labeled secondary genome would not become stabilized until the secondary DNA has been allowed to synthesize some necessary protein of its phenotype.

Finally, a further interpretation of the nature of the individually specific protein can be made which envisages its role in the stabilization of the secondary genome as neither replication activator nor essential gene product. In order for an infective center to be scored as liberating the three secondary genetic markers h, r_7^+, and r_1 on the K + B/4/2 bacterial indicator mixture, it is necessary that h and r_1 markers issue from the infected cell in phage particles which also bear the r_7^+ marker, i.e., the linkages h—r_7^+ and r_7^+—r_1 must be preserved. Now it is not excluded that the material continuity of the T-even phage chromosome depends on protein links between DNA molecules. In this case, it might be possible that the labeled secondary phage DNA actually replicates in superinfected bacteria in the presence of chloramphenicol without manifesting any stabilization to radioactive decay. For such replication would then generate *partial replicas* of the parental chromosome which do not preserve the long range linkage of the secondary markers. Radiochemical destruction of the secondary parental DNA before or after the onset of such replication would then destroy the last trace of linkage, rendering it very unlikely that the required secondary genotypes are reconstituted from the vegetative pool in which these partial replicas represent a minority parent (Visconti & Garen, 1953; Visconti & Delbrück, 1953). This hypothesis, however, predicts that stabilization of a *single* marker of the labeled secondary phage should occur in the presence of chloramphenicol. We have followed the survival of the secondary r_1 marker in the experiment presented in Fig. 2, but, as was pointed out already, the slow rate of decay of single markers makes such stabilization difficult to establish. Our data, in any case, do not exclude the possibility that stabilization of the secondary r_1 marker occurs in the presence of the antibiotic.

Before closing, mention must be made of an experiment of Thomas (1959), which was designed to answer the same question to which we have addressed ourselves in this work. Thomas infected *E. coli* bacteria with the h^+ wild type of phage λ, then made a secondary infection with an h mutant at various later times, and finally scored the genotype ratio h^+/h among the phage progeny. Since, unlike in the case of T-even phages, no serious exclusion of secondary phages occurs in λ-infected bacteria, the increase with time in the h^+/h ratio among the progeny measures the number of vegetative h^+ phages present in the intrabacterial pool at the time of the secondary infection. It could be shown by means of this technique that the synthesis of an "early" protein is also required for the genetic replication of the λ phage, and that once this protein has been made, a rather limited, twofold multiplication of the primary h^+ genome can take place in the presence of chloramphenicol. It was found, furthermore, that if the secondary h genome is introduced into the cell only after protein synthesis has just been inhibited, then the secondary h genome participates along with the primary h^+ genome in the ensuing limited replication in chloramphenicol. Thomas thus concludes that the "early" protein of phage λ is not "individually specific," in that the protein induced by the primary h^+ also serves for the replication of the secondary h phage.

It will require further work to decide whether the apparently contradictory conclusions of Thomas and our experiments derive from differences in the two phage systems employed, or whether the two experimental approaches—genetic replication and decay stabilization—actually pertain to different aspects of DNA function.

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Environment-Induced Changes in the Base Ratios of Ribonucleic Acid of *Escherichia coli*

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Differences have been found in the base ratios of the total RNA of *E. coli* when it was grown on different media.

1. Introduction

A great deal of evidence supports the view that DNA contains the information which determines the specificity of proteins. In addition several lines of evidence involve RNA in protein synthesis (Fraenkel-Conrat, 1956; Brachet, 1957; Zamecnik, Stephenson & Hecht, 1958); thus it has been suggested that RNA serves as an intermediary in the transfer of genetic information contained in DNA to proteins. Various models have been proposed to account for the transfer of information from DNA to RNA (Crick, Griffith & Orgel, 1957).

An apparent paradox has arisen; although the DNA base ratios of various gram negative bacteria are different, their RNA base ratios are very similar and in some cases identical (Belozersky, 1957; Lee, Wahl & Barbu, 1956; Belozersky & Spirin, 1958). Various explanations have been advanced (Crick, 1959; Sinsheimer, 1959; Sueoka, Marmur & Doty, 1959), and we should like to offer another (although not exclusive) alternative.

First of all, we suggest that the similarity in RNA base ratios of the various gram negative bacteria tested resulted from their growth under similar or identical conditions. Under these similar environmental states the various organisms are called upon to make similar proteins and presumably similar kinds of RNA. This relationship, if true, suggests that, if environmental states were drastically altered for one organism, the cell might respond by making different RNA molecules and consequently different proteins. These suggestions are supported by three reports by Astrachan & Volkin (1958), Wade & Morgan (1957) and Spahr & Tissières (1959). In each of these studies *E. coli* was grown on a medium other than glucose and salts. Volkin & Astrachan grew their bacterium on a peptone medium, while Wade & Morgan grew *E. coli* on a "casein-casein-yeast extract" medium; Spahr & Tissières grew it on a tryptone-yeast extract medium. The base ratios of the total RNA of *E. coli* (as reported in the first two papers) were different from the values reported by both Lombard & Chargaff (1957) and Belozersky & Spirin (1958). Lombard & Chargaff (1957) grew *E. coli* on a glucose-salts medium. However, it should be noted that Spahr & Tissières were dealing with the RNA contained in the ribosomal particles; therefore their values are not directly comparable to those of Lombard & Chargaff (1957). The experiments reported here demonstrate a significant alteration of the base ratio of the total RNA of *E. coli* B when grown under different environmental conditions.

2. Methods

E. coli B was grown on the following media at 37°C: nutrient broth (Difco), 0·8%, and yeast extract (Difco), 0·1%; the glucose-salts medium contained 0·8% K_2HPO_4, 0·1% $MgSO_4$, and 0·1% $(NH_4)_2SO_4$, and was adjusted to pH 6·8; the glucose concentration was 1%. The glucose-grown cells were harvested after 18 to 20 hr growth, while the nutrient broth-yeast extract-grown cells were harvested after 12 hr growth; both populations were near the end of their log phase. The cells were harvested in a Sharples centrifuge, washed twice with distilled water, resuspended in 100 ml. distilled water, and the RNA mononucleotides were isolated by the method of Volkin & Astrachan (1956) with the following modifications. The total nucleic acid fraction was dialyzed against buffer for 18 hr and then against distilled water for 3 hr before hydrolysis with alkali. After alkaline hydrolysis polymerized DNA was precipitated by addition of concentrated HCl. Three solvents were used for the separation of mononucleotides on Whatman No. 1 filter paper: *iso*propanol-HCl-H_2O for separating uridylic and cytidylic acids (Wyatt, 1951); *iso*propanol-H_2O (NH_3 in vapour phase) for the determination of guanylic acid (Markham & Smith, 1952); and *iso*butyric-NH_3 for the separation of adenylic and cytidylic acids (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950). The mononucleotide spots were located on the paper using an ultraviolet lamp, cut out and eluted with 4 ml. of 1 M-phosphate buffer, pH 7, in the case of the *iso*butyric and *iso*propanol-NH_3 solvents, and with 4 ml. of distilled water in the case of the *iso*propanol-HCl solvent. The test tubes containing the eluting spots were incubated for 16 hr at 37°C. The absorption spectrum of each tube was read in a Beckman model DU spectrophotometer from 250 to 280 mμ. Optical density units were converted to μg/ml. of nucleotide using the ϵ values of Elson, Gustafson & Chargaff (1954). About 15 determinations were made on each batch of *E. coli* grown on either medium for each nucleotide. Deoxyribose determinations were carried out using the diphenylamine reagent (Dische, 1955).

3. Results and Discussion

The results of the analyses carried out in this investigation are presented in Table 1. Table 1 contains the base ratios of the total RNA of *E. coli* B when grown in the two kinds of environments. The RNA base ratio values reported here for glucose-grown *E. coli* are essentially identical to those values reported by Lombard & Chargaff (1957) and Belozersky & Spirin (1958), while the results with nutrient broth-yeast extract-grown cells are different. The most marked differences are the guanylic to adenylic, and uridylic to adenylic ratios. These ratios are like those reported by Astrachan & Volkin (1958) and Wade & Morgan (1957) for the total RNA of *E. coli* and Spahr & Tissières (1959) for the 30 s and 50 s particles of *E. coli*. Although Spahr & Tissières did not analyze for the total RNA, it is difficult to imagine that such a small proportion of the RNA would so markedly influence the ratios of bases contained in such a large fraction of the total RNA of the cell. In fact the data of Bolton (1960) indicate that the soluble RNA of *E. coli* has a much higher guanylic to adenylic and uridylic to adenylic ratio than does the particulate RNA fraction.

The data in this paper do not resolve the problem of how one might reconcile the wide range of DNA base ratios in gram negative bacteria with a rather narrow range of RNA base ratios. However, we may explain the results by making the following assumptions:

1. Under any one set of environmental conditions a small portion of the DNA is involved in RNA synthesis.

2. This involvement may manifest itself in a quantitative way; i.e., some RNA molecules are produced more often than others. Under these conditions, it would be highly unlikely that the RNA composition of a cell would ever reflect the DNA composition.

The results reported here indicate that environmental states influence the biosynthesis of RNA in *E. coli*. This situation may be related to altered protein synthesis.

TABLE 1

Nucleotide composition of the total RNA of E. coli expressed in moles per 100 moles of the four nucleotides

The values under A represent the mean of twelve to fifteen separate analyses for each nucleotide. They are presented with the errors expressed as average deviation from the mean. The figures under column B represent the average molar ratios derived from three separate experiments.

	Glucose grown		Nutrient broth-yeast extract grown	
	A	B	A	B
Adenylic acid	26·6 ± 0·5	26·1 ± 0·7	24·0 ± 0·4	23·9 ± 0·6
Cytidylic acid	22·9 ± 0·7	24·3 ± 0·5	22·1 ± 0·7	24·0 ± 0·7
Uridylic acid	20·4 ± 0·6	20·3 ± 1·0	23·3 ± 0·3	21·3 ± 0·6
Guanylic acid	30·1 ± 0·2	29·3 ± 0·5	30·5 ± 0·3	30·9 ± 0·6
Purines/pyrimidines	1·22	1·24	1·20	1·21
†6-Am/6-K	0·98	1·01	0·86	0·92
‡(A + U/G + C)	0·89	0·87	0·90	0·82

†6-Am: 6-amino nucleotides (adenylic and cytidylic acids)
6-K: 6-keto nucleotides (guanylic and uridylic acids)
‡A, G, C, and U stand for adenylic, guanylic, cytidylic and uridylic acids respectively.

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The "Cross-β" Structure in Polypeptides of Low Molecular Weight

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Observations of extended polypeptide chains whose direction is perpendicular to the fibre axis ("cross-β" structures) have hitherto been confined to proteins. This structure has now been found in some synthetic polypeptides of rather low molecular weight, and is especially well-developed in poly-β-*n*-propyl-L-aspartate films cast from warm dichloroacetic acid, oriented by stroking during the drying process.

Infrared spectra of such films recorded with polarized radiation show that the C=O and NH bonds of the peptide group are approximately along the direction of stroking, as in the α-helix. However, the band frequencies are those characteristic of the β (extended) configuration. X-ray diffraction photographs show the characteristic reflections of this form. It is quite clear, however, that the chain direction is normal to the fibre axis, confirming the interpretation of the infrared spectra. Double orientation of suitable prepared specimens has enabled a fairly complete determination of the unit cell to be made. A monoclinic cell containing four chains (eight peptide residues) is proposed with

$$a = 9{\cdot}57 \text{ Å}, \; b = 6{\cdot}79 \text{ Å (chain axis)},$$
$$c = 25{\cdot}08 \text{ Å}, \; \beta = 96°.$$

The space group may be $P2_1$, and it is suggested that not all residues are equivalent. The evidence indicates that the chains in the hydrogen-bonded sheet alternate in direction (antiparallel sheet).

1. Introduction

The term "cross-β" appears to have been used first by Astbury, Dickinson & Bailey (1935) to indicate a protein structure whose X-ray diffraction pattern was characterized by a reflection from planes approximately normal to the direction in which the specimen had been stretched or rolled, spaced about 4·7 Å apart. This pattern was interpreted as indicating the presence of extended polypeptide chains with their axes normal to the direction of stretching (i.e. normal to the fibre axis) connected to neighbouring chains by hydrogen bonds in the same way as in the more familiar extended chain (β) structure, in which, however, the chains are parallel to the fibre axis. The formation of the cross-β pattern in epidermal protein has been described in detail (Rudall, 1946, 1950, 1952).

Additional evidence for a structure of the cross-β type was found by Ambrose & Elliott (1951*a*), who reported that fibrous insulin, oriented by stroking out the gel formed in formic acid, showed infrared dichroism which indicated that the C=O and NH peptide bonds were approximately along the direction of stroking. At the same time, the frequency of the C=O band corresponded to that found in β (extended chain) synthetic polypeptides and proteins, and the X-ray diffraction photograph

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had a meridional arc corresponding to an interplanar distance of 4·7 Å. The most complete evidence for a cross-β structure has been found by Parker & Rudall (1957) in the silk of the egg-stalk of the green lace-wing fly *Chrysopa flava*. The X-ray diffraction pattern of this silk is so well developed that it is certain that the *b* or chain axis (period 6·95 Å) is normal to the fibre axis, as is the *c* axis, whereas the *a* axis (period 9·44 Å) is parallel to the fibre axis. In the usual β structure of silks, similar axial repeats are found, but the *b* axis is parallel to the fibre axis. The infrared dichroism of *Chrysopa* egg stalks is of the same character as that of fibrous insulin referred to above and the frequency of the peptide C=O band, 1625 cm^{-1}, is in the region characteristic of extended polypeptide chains (not of α-helices). On stretching the egg-stalk, the structure changes to the normal β type.

Zubay (1959) has suggested that the meridian reflection corresponding to 4·7 Å seen in the X-ray diffraction patterns of some suitably heat-treated proteins does not indicate the presence of near-extended polypeptide chains. He observes that the $10\bar{1}5$ reflection from a hexagonally packed arrangement of α-helices (that is, the first reflection on the 5·4 Å layer line of an 18/5 α-helix) would correspond to a spacing of 4·7 Å if the first equatorial reflection were due to a spacing of 10·1 Å, approximately the value observed in some (but not all) cross-β structures. This is undoubtedly true; in the diffraction pattern of poly-γ-methyl-L-glutamate in the oriented α-helix form the first equatorial reflection and the first reflection on the 5·4 Å layer line correspond respectively to 10·34 and 4·77 Å (Bamford, Brown, Elliott, Hanby & Trotter, 1952). In cases where the orientation is too low to allow the origin of a 4.7 Å reflection to be deduced with certainty, the frequency of the peptide C=O band (Amide I) in the infrared spectrum gives some indication (Ambrose & Elliott, 1951*b*; Elliott, 1953). When this band does not move from *ca* 1660 to 1630 cm^{-1} as a result of the treatment which produces the 4·7 Å diffraction arc, it is doubtful whether the polypeptide chains have adopted a near-extended, β configuration, though a small fraction of them may have done so without an appreciable peak appearing at 1630 cm^{-1}. In the cases of *Chrysopa* silk and insulin referred to above, it is clear that the structure is not that of an α-helix, and very strong reasons exist for describing them as cross-β structures.

We have recently found very good evidence for a cross-β structure in some synthetic polypeptides. In one instance (poly-β-*n*-propyl-L-aspartate) the state of orientation of the polymer has allowed the unit cell of the crystalline part to be determined with greater certainty than in the case of any other polypeptide in either the normal β or the cross-β form.

2. Experimental

The synthetic polypeptides were prepared in the usual way by polymerization of the N-carbonic anhydride. Details of the preparation will be given in a more complete account of polymers made from aspartic acid.

The reduced viscosities were measured in dichloroacetic acid (in which solvent, at low concentration, the polymers appear to form random solvated coils). The values are as follows. (Concentration 0·5% w/v).

Polymer	$\eta_{sp/c}$ (dl.g)
Poly-β-*n*-propyl-L-aspartate	0·095
Poly-β-benzyl-L-aspartate	0·121
Poly-γ-benzyl-L-glutamate	0·064

The infrared spectra in the 6μ region were recorded on a Grubb-Parsons S1 instrument, with a reflecting microscope and an arrangement for rocking the specimen (Elliott, 1959).

The frequencies of the NH stretching bands were measured with a Perkin-Elmer 12A instrument with a lithium fluoride prism. The wave-numbers of the amide bands are given in Table 1.

Table 1

	Poly-β-*n*-propyl-L-aspartate		Poly-β-benzyl-L-aspartate		Poly-γ-benzyl-L-glutamate
	from chloroform	from dichloroacetic acid	from chloroform unheated	from chloroform heated at 160°C	from chloroform
	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}	cm^{-1}
NH stretching mode	3305 m.	3285 m.	3315 †m.	3285 m.	3296 m.
Weak N—H	3075 v.w.	3085 v.w.	3076 v.w.	3080 v.w.	3075 v.w.
Ester C=O	1740 s.	1738 s.	1737 s.	1730‡ s.	1735 s.
Band near 1700 cm^{-1}	—	1700 w.	—	—	1692 w.
Amide I					
ω-form	—	—	—	1675 s.	—
α-helix	1662 v.s.	1660 w. sh.	1665 v.s.	—	1655 m.
β	—	1637 v. s.	—	1632 m.	1623 s.
Amide II					
ω-form	—	—	—	1541 m.	—
α-helix	1554 m.	1552 w. sh.	1561 m.	—	1551 w.
β	—	1530 m.	—	1535 w.	1525 m.

†The frequency of the N—H stretching band varies somewhat from one polymer preparation to another; it may be dependent on molecular weight.

‡Attributed to ω-configuration.

v.s. = very strong, s = strong, m = medium, w = weak, v.w. = very weak, sh. = shoulder.

3. Results

Poly-β-n-propyl-L-aspartate

This polymer was oriented by stroking out a concentrated solution in chloroform. The spectrum of a specimen prepared in this way (Fig. 1(a)) shows the usual features of an oriented folded (α-helix) form (Ambrose & Elliott, 1951*b*; Elliott, 1953). The peptide C=O stretching band at 1662 cm^{-1} and the N—H stretching band at 3305 cm^{-1} have parallel dichroism (with respect to the direction of stroking) and the Amide II band at 1540 cm^{-1} is of perpendicular character. When this specimen was heated *in vacuo* for about an hour at 160°C the dichroism almost vanished, and the C=O band appeared at a lower frequency, 1637 cm^{-1}, suggesting that the polypeptide had changed to the unoriented, extended β form. The NH stretching band was also lowered in frequency to 3285 cm^{-1}. Fig. 2 shows the spectrum of an unoriented specimen cast from chloroform solution (a), heated as above (b), and recast from chloroform without heating (c). It is evident that no irreversible change has been produced, for in (a) and (c) the frequencies do not differ significantly. This removes the possibility that chemical changes produced by heating are responsible for the frequency differences between (a) and (b). It also excludes the explanation, rather implausibly suggested in connection with some earlier work in this laboratory, that a fraction of the polymer might have evaporated during the heating, and that the remaining component might have properties different from those of the original polymer (Blout & Asadourian, 1956).

When the same preparation of poly-β-*n*-propyl-L-aspartate was dissolved in dichloroacetic acid and the solution was stroked out to dryness at about 60°C, the oriented specimen was obtained whose spectrum is shown in Fig. 1(b). The frequencies of the chief amide bands are identical with those in the spectrum of the film cast from chloroform and subsequently heated, but the dichroism shows that the peptide C=O and NH bonds are approximately in the direction of stroking. If the polyptide chains are extended, the structure must be of the "cross-β" type.

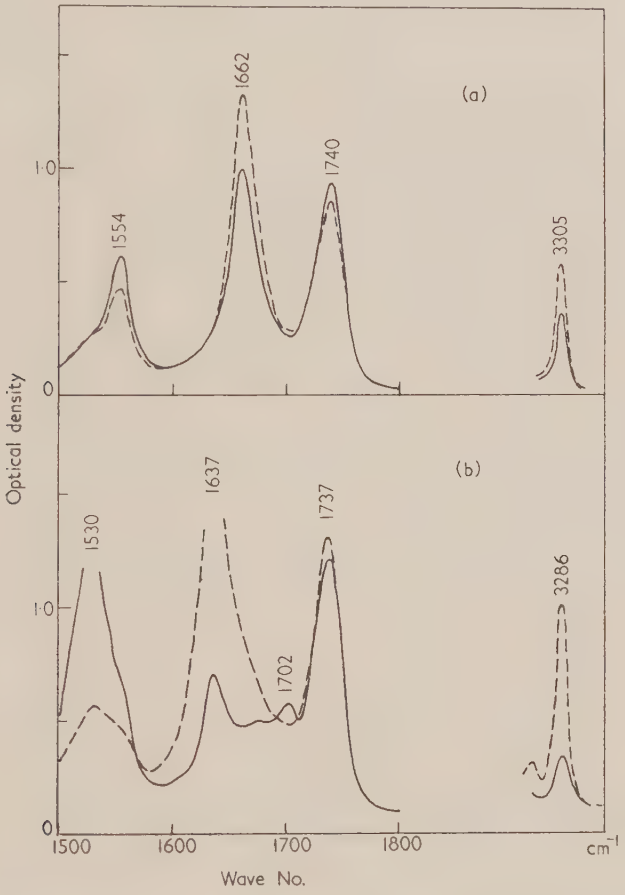


FIG. 1. Infrared spectra (polarized radiation: full line with E-vector perpendicular to fibre axis; broken line with E-vector parallel to fibre axis).

(a) Poly-β-*n*-propyl-L-aspartate film cold-cast from chloroform and stroked. (b) The same hot-cast from dichloroacetic acid.

It is of interest to note that in the infrared spectrum shown in Fig. 1(b) a distinct though weak band appears at about 1700 cm^{-1}, with dichroism opposite in character to that of the peptide carbonyl stretching band. A band of this kind in the region 1685 to 1700 cm^{-1} has been reported in β structures (Elliott & Malcolm, 1956; Bamford, Elliott & Hanby, 1956), and this is doubtless the same band. Another feature of Fig. 1(a) is that in the α-helix spectrum the ester C=O band at 1740 cm^{-1} shows weak perpendicular dichroism. In spectrum (b) this has changed to an equally weak parallel dichroism.

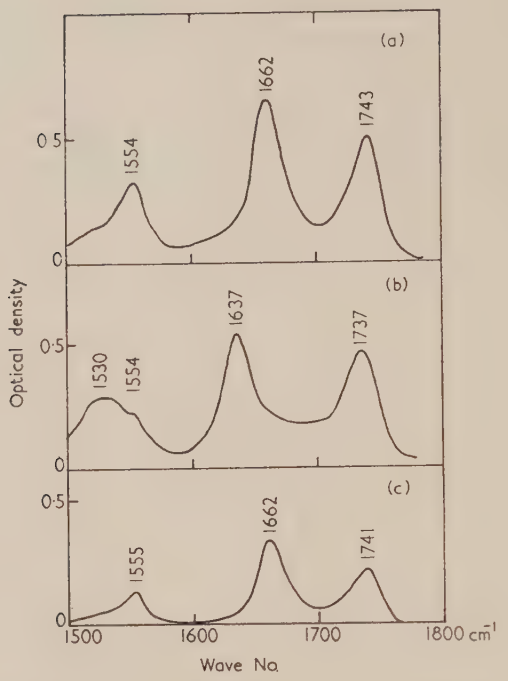


FIG. 2. Infrared spectra of poly-β-*n*-propyl-L-aspartate.

(a) Cast from cold chloroform. (b) Heated 1 hr at 160°C *in vacuo*. (c) Re-cast from cold chloroform.

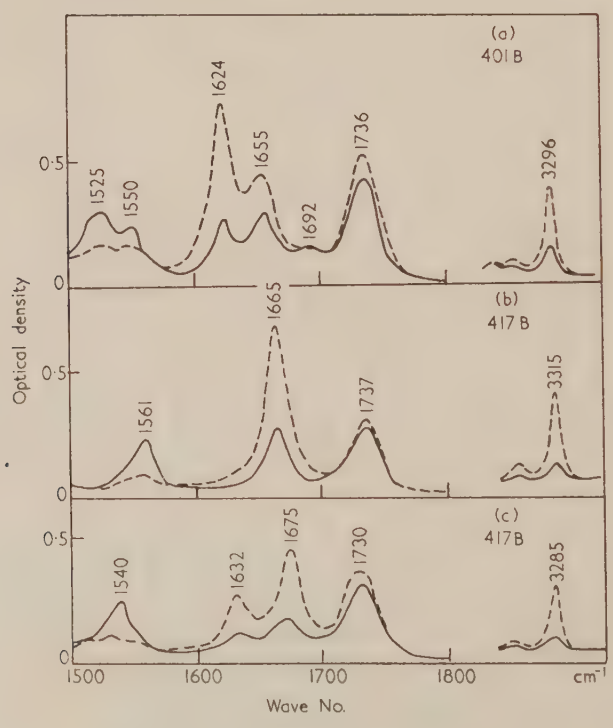


FIG. 3. Spectra in polarized radiation as in Fig. 1.

(a) Poly-γ-benzyl-L-glutamate cold-cast from chloroform and stroked. (b) Poly-β-benzyl-L-asparatate cold-cast from *m*-cresol and stroked. (c) As (b), but heated 1 hr at 160°C *in vacuo*.

Very clear evidence that the structure is of the cross-β type is obtained from X-ray diffraction. Specimens prepared as described above, mounted with the direction of stroking parallel to the axis of a cylindrical camera (as is usual with fibre photographs), give a diffraction photograph with a strong arc corresponding to 4·76 Å on the meridian, and a strong 13 Å reflection on the equator. The former reflection corresponds approximately to the distance between hydrogen-bonded polypeptide chains in a sheet, and the latter to the distance between consecutive sheets (the side-chain spacing). Some specimens, prepared in the same way, have a high degree of "double orientation" (Bunn & Garner, 1947) and show beyond doubt that the b axis of the unit cell, corresponding to the near-extended chain axis repeat of 6·79 Å, is in the plane of the film but perpendicular to the direction of stroking. Of the many diffraction photographs taken with a large range of orientations of specimen to X-ray beam, three are reproduced in Plate I. The "vertical" on Plates I(a) and I(b) corresponds to the direction of stroking. With the X-ray beam parallel to the film surface of the specimen a very strong reflection from the (002) planes appears corresponding to 13 Å. This reflection is weak almost to vanishing in Plate I(a) with the X-ray beam normal to the film. In a photograph taken with the X-ray beam along the direction of stroking (the a axis), as shown in Plate I(c), the reflections produced in the equatorial plane of Plates I(a) and I(b) are seen, and the relation between the specimen and the planes from which the reflections arise is evident.

Most of the reflections can be indexed with a simple monoclinic cell containing one chain, with $a = 4{\cdot}78$ Å, $b = 6{\cdot}79$ Å, $c = 12{\cdot}54$ Å, $\beta = 96°$. There are, however, several reflections which indicate quite clearly that the sides a and c of this cell require to be doubled, indicating that at least two types of chains are present. We shall discuss the structure in terms of a unit cell containing four chains, in which $a = 9{\cdot}57$ Å, $b = 6{\cdot}79$ Å (chain axis), $c = 25{\cdot}08$ Å, $\beta = 96°$. The relation between specimen and unit cell is shown in Fig. 4. Only one reflection (spacing 19 Å) cannot be

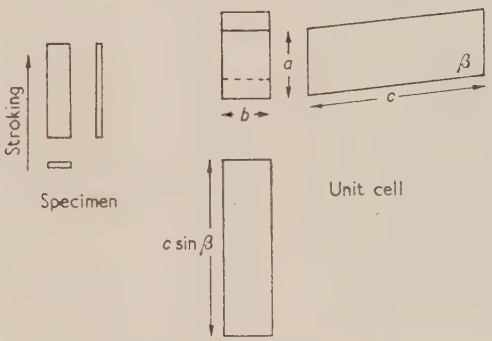


FIG. 4. Relation between specimen and unit cell in cross-β form of poly-β-n-propyl-L-aspartate. $a = 9{\cdot}57$ Å, $b = 6{\cdot}79$ Å, $c = 25{\cdot}08$ Å, $\beta = 96°$.

indexed with this unit cell; its significance is discussed below. The calculated and measured values of the density are 1·287 and 1·24 g/ml., respectively.

The spacings, calculated and observed, are shown in Table 2. The high degree of double orientation enabled many more to be observed than would otherwise have been possible. By taking account of the intensity of the reflections produced with different orientations of the specimen with respect to the X-ray beam, ambiguities in the indexing have been almost entirely avoided in the b^*c^* plane.

It has been possible in this way to show that the 007 and 009 reflections are correctly indexed, although from the magnitude of the spacing alone they could also be indexed as 016 and 018. On a photograph taken with the *a* axis along the cylindrical

TABLE 2

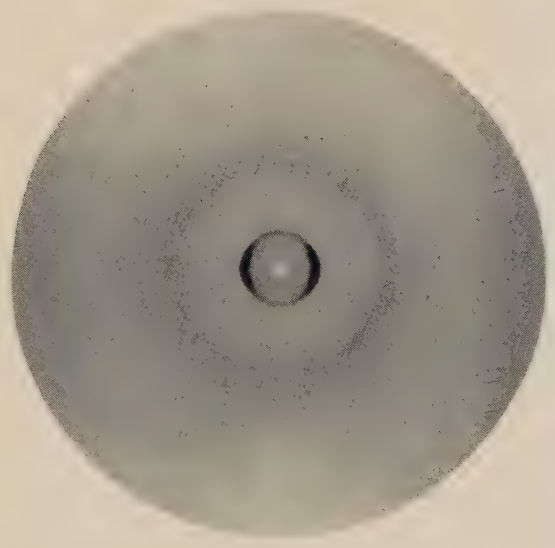
Poly-β-n-propyl-L-aspartate: observed and calculated spacings

hkl	$d_{(obs)}$ Å	$d_{(calc)}$ Å	other possible indices
002	12·9	12·47	—
Diffuse	10·5	—	—
005	5·02	4·99	—
006	4·18	4·16	—
007	3·56	3·56	—
008	3·10	3·12	—
009	2·77	2·77	—
0010	2·51	2·49	—
0011	2·27	2·27	—
012	6·02	5·96	—
014	4·59	4·59	—
020	3·32	3·40	—
022		3·27	—
024	3·02	2·98	—
026	2·65	2·63	
028	2·28	2·30	—
060	1·131	1·131	—
100?	9·0	9·52 diffuse	—
11$\bar{2}$	5·27	5·17	110, 10$\bar{4}$
130	2·19	2·20	13$\bar{2}$
200	4·77	4·76	—
202	4·34	4·30	—
210	3·90	3·90	21$\bar{2}$, 20$\bar{4}$
212	3·59	3·63	204, 21$\bar{4}$
214	3·24	3·18	206
220	2·76	2·76	21$\bar{6}$, 22$\bar{2}$
230	2·05	2·04	23$\bar{2}$
320	2·33	2·32	32$\bar{2}$
330	1·85	1·84	33$\bar{2}$
400	2·38	2·38	—
410	2·26	2·25	41$\bar{2}$, 402
420	1·95	1·95	42$\bar{2}$
430	1·63	1·64	43$\bar{2}$
600	1·58	1·59	60$\bar{2}$, 604

camera axis and the X-ray beam at an angle of 76° to the normal of the specimen film, these reflections appear as equatorials, so that the corresponding reciprocal lattice points are in the b^*c^* plane. The reflections are much stronger on that side of the centre where 007 and 009 (but not 016 and 018) would be expected to appear most strongly. On photographs taken with the *b* (chain) axis parallel to the cylindrical camera axis and with suitable orientation around the camera axis, the reflections in question again appear as equatorials. It follows that the corresponding reciprocal

(a)

(b)



(c)

PLATE I. X-ray diffraction photograph of cross-β form poly-β-*n*-propyl-L-aspartate (Cu Kα).
(a) Direction of stroking vertical, X-ray beam normal to surface (3 cm cylindrical camera).
(b) As (a), but X-ray beam parallel to film surface.
(c) X-ray beam along direction of stroking; plane of film vertical (4 cm flat film).

[*To face page* 282

lattice points are in the a^*c^* plane. These observations taken together show that the reflections must be indexed as $00l$. The $h00$ reflections with even values of h are remarkably sharp for a polymeric material; this leads to a very good agreement between observed and calculated values of these spacings (Table 2). In contrast to this, the $00l$ reflections are broad, and the agreement between observed and calculated values of the spacings is poor for 002. We have preferred to base the value of c on spacings other than this one, believing that the observed value of the 002 spacing may differ considerably from that which would be observed with an infinite crystal. We can be fairly certain that the molecular transform will increase from the 002 reciprocal lattice point to the origin monotonically, and that in consequence the sense of the gradient at that point will be such as to cause the maximum intensity of a broad reflection to appear nearer to the origin than the reciprocal lattice point. The observed value of the 002 spacing will therefore be too great, as we have found.

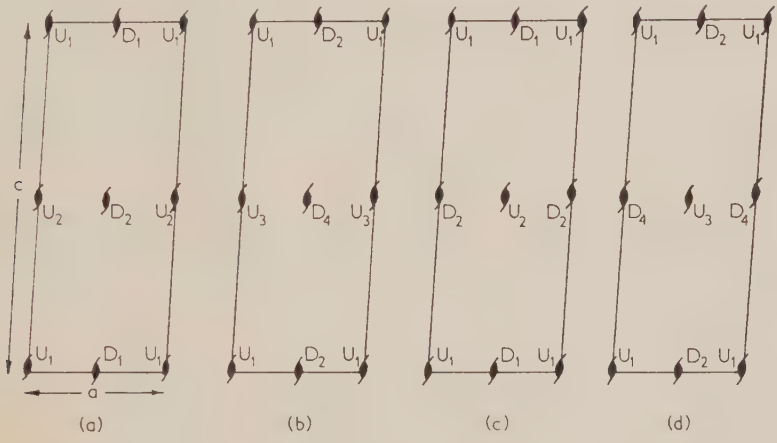


FIG. 5. Possible unit cells for cross-β form of poly-β-*n*-propyl-L-aspartate. Chains U_n and D_n differ only in chain sense.

The doubling of the a axis is naturally explained by supposing that there is an alternation of chain sense in the sheets formed by hydrogen bonds between chains, so that the repeat of pattern is twice the distance between neighbouring chains. The possibility that this arrangement is occasionally interrupted is discussed below. The reason for giving the c axis the value 25·08 Å instead of 12·54 Å comes from the observation of the reflections 007 and 009. These and other $00l$ reflections with l odd can appear only if the repeat of pattern of electron density, projected on the normal to the $00l$ planes, is 25·08 Å (or a multiple of this length), and this implies that consecutive hydrogen-bonded sheets appear different when projected in this way. This requirement cannot be met by an anti-parallel arrangement of otherwise identical chains along the c axis. A difference of the required kind could be introduced by rotating chains in alternate hydrogen-bonded sheets around the b axis, but this would lead to an unfavourable arrangement of hydrogen bonds, as discussed in the next section. It appears necessary to suppose that different chain configurations exist. The great intensity of the 200 and 002 reflections shows that the chains all lie on or very near the lattice points formed by the simple cell of sides $a/2$ and $c/2$. Fig. 5 shows four arrangements belonging to the space group $P2_1$, all of which require the individual chains to have a twofold screw axis, and would be compatible with the experimental evidence. The unit cell contains two asymmetric units, each of which

would consist of four peptide residues, one from each of the differently marked chains. The sense of the peptide sequence is the same in all chains designated U_n, and oppositely directed in the D_n chains. In arrangements (a) and (c) there are only two configurations of residue, since U_1 and D_1 differ only in position and orientation. In (b) and (d), however, there are four different residue configurations.

We must suppose that the structure of the polymer is mainly settled by the need to satisfy the inter-chain hydrogen bonds. This decides the length of the *a* and *b* axes, the structure of each hydrogen-bonded sheet and the positions of the β-carbon atoms. These sheets then pack as well as possible to give minimum free energy to the molecular side-chains. In the case of poly-L-alanine, since the position of the β-carbon atom is fixed, all residues must be identical except that rotation around the αC-βC bond allows the possibility of different positions of the hydrogen atoms in the methyl groups, a difference which would hardly affect the X-ray diffraction pattern. If the chains in the hydrogen-bonded sheet are anti-parallel, the most favourable situation occurs when these chains are related by a twofold screw axis parallel to *a*. Identity of all residues (except for the small difference referred to above) may then be expected to result in a form of packing in which each residue is similarly surrounded. With asymmetric residues the unit cell can have only rotation and screw axes (mirror planes, glide planes and centres of symmetry are excluded since they change L-residues into D-) and with anti-parallel sheets the resulting crystal has the space group $P2_12_12_1$ as suggested by Marsh, Corey & Pauling (1955) for the pseudo unit cell of tussah silk and poly-L-alanine. It is clear from the monoclinic cell of poly-β-*n*-propyl-L-aspartate that the space group is not $P2_12_12_1$, and the molecular chains evidently pack in a different way from those of poly-L-alanine.

In crystals, the state of minimum energy is usually reached when identical chemical units have identical configurations and environments, but exceptions occur. It is easy to see, in a general way, that the side chains of a β-polypeptide might be one of these exceptions. Since the separation of the β-carbon atoms in the *ab* plane is fixed by the hydrogen-bonding forces, it could happen that identity of all side chains would lead to the inter-atomic distances being larger, on the whole, than those corresponding to normal van der Waals forces. Because the force of attraction falls off rapidly with increasing distance once the van der Waals distance is exceeded, it might be energetically profitable for some chains to interact more closely, and others less closely, than for all to have the same configuration. On the other hand, in an anti-parallel sheet at least, the operation of the twofold screw along the chain axis and of the twofold screw axis parallel to *a* could in some circumstances cause side-groups on an "up" chain to come opposite side-chains on a neighbouring "down" chain, causing more interference (and higher energy) than would have been the case had the two chains adopted different configurations. The conditions necessary for this are difficult to formulate and might perhaps not occur in side chains where free rotation could occur around every bond. They would certainly occur with suitable constraints on this rotation.

As mentioned earlier, the $h00$ reflections with even values of h are remarkably sharp for a polymer, which indicates that the sequence of chains 4·78 Å apart is very well-developed. However, some of the reflections which indicate that *a* is 9·57 Å and not 4.78 Å are diffuse, particularly those with $h = 1$ (one of these may be seen on Plate I(a)). The diffuseness may be caused in part by the near-coincidence of reflections 110 and 112 but seems not to arise entirely in this way.

With a chain repeat length of 6·79 Å there is little difference between the linearity

of the N—H . . . O atoms in parallel and in antiparallel hydrogen-bonded sheets. It is therefore possible that the diffuseness of the reflections with $h = 1$ is caused by an occasional interruption of the regular alternation of chain sense. It is, however, curious that the reflections with $h = 3$ are not particularly diffuse. It is conceivable that some measure of randomness in chain sense could more easily occur in arrangements (a) and (c) rather than in (b) or (d) (Fig. 5). We have examined models of the β form of poly-β-*n*-propyl-L-aspartate with the above conclusions in mind, but have been unable to decide which of the various possibilities is the most likely. It is, however, clear that the side-chains cannot be fully extended or even nearly so, for this would result in much too high a density midway between the two sheets.

One reflection is not accounted for by the unit cell discussed above. The corresponding reciprocal lattice point is in the b^*c^* plane, at a distance from the origin 1/19 Å^{-1}, approximately equally far from the b^* and the c^* axes. Since it appears on photographs taken with radiation which has been reflected from a quartz crystal, it cannot be ascribed to white radiation. It is just possible that it is connected in some way with the actual length of the molecules, as distinct from the chain repeat length, though in view of the range of molecular weight which may occur during polymerization this would be surprising. It is intended to examine the region of reflection corresponding to long spacings with a camera now being constructed.

Poly-β-benzyl-L-aspartate

When specimens of poly-β-benzyl-L-aspartate of not too low molecular weight are prepared by stroking from solution in chloroform, an oriented α-helix structure is obtained. This structure is not stable, however; on heating to about 160°C a new form appears (which we have called the ω-form) (Bradbury *et al.*, 1959). When the molecular weight of the polymer is low ($\eta_{sp}/c = 0{\cdot}21$) an oriented α form is obtained by stroking from solution in *m*-cresol and the usual α-type dichroism is found, as shown in Fig. 3. The peptide C=O band appears at 1665 cm^{-1}, which is rather high for an α-polypeptide made from a single optical isomer of an amino acid (Elliott 1953). After heat-treatment (Fig. 3(c)), this C=O band has two peaks at 1675 and 1632 cm^{-1}, each having parallel dichroism. The high-frequency band indicates the presence of the ω-form, and the other weaker band would appear to indicate a cross-β structure. Confirmation of this has now been found in X-ray diffraction photographs, and the meridian reflection 4·7 Å which characterizes this structure can be distinguished from other, stronger reflections in the same region which are produced by the large fraction of material in the ω-form.

Poly-γ-benzyl-L-glutamate

The first synthetic polypeptide in whose spectrum really high dichroic effects were observed was poly-γ-benzyl-L-glutamate (Ambrose & Elliott, 1951*b*). With a polymer of high molecular weight the α-helix form is obtained from a variety of solutions. When, however, the molecular weight is low, films cast from chloroform and other solvents appear to consist of mixtures of α-helix and β-forms (Blout & Asadourian, 1956); this is found with many other polypeptides (Bamford, Hanby & Happey, 1951; Robinson & Bott, 1951). The spectrum of an oriented sample ($\eta_{sp}/c = 0{\cdot}064$) made by stroking from solution in chloroform (Fig. 3(a)) shows that the polymer contains a proportion of the oriented α-helix form (C=O band at 1655 cm^{-1}). However, the strongest C=O peak, which also has parallel dichroism, is at 1624 cm^{-1} and this is characteristic of the cross-β structure.

4. Discussion

The cross-β structures described here have been produced in two ways. With a suitable solvent-polymer combination, this oriented structure may be formed by stroking out a drying solution or gel, without heat treatment. We may suppose that at some stage micelles are formed by lateral association of extended chains. In the case of poly-γ-benzyl-L-glutamate the degree of polymerization was about twenty; the reduced viscosity of the poly-β-*n*-propyl-L-aspartate was higher than that of the glutamate polymer and this may perhaps correspond to a higher degree of polymerization. In order to produce a micelle sufficiently anisotropic to orient under the shearing forces (since the chain direction in the polymer is normal to the direction of stroking) it would appear that the number of associating molecules must greatly exceed twenty. This type of association is similar to that found in crystals of some simple substances in which the molecules are long and thin, and has been suggested for fibrous insulin (Ambrose & Elliott, 1951*a*). Unless the 19 Å reciprocal lattice point appearing in the b^*c^* plane (as yet not satisfactorily indexed) should prove to be connected with a long-period folding of polypeptide chains there appears to be no difference between the cross-β structure of poly-β-*n*-propyl-L-aspartate and that of the more familiar type of β structure except that of habit. In this respect the cross-β structure of *Chrysopa* silk, in which there appears to be secondary folding of the β-chains, is different.

The other way of producing the cross-β structure is also interesting. Here a well-oriented α-helix form is produced and by heating the solid film a partial (though not a complete) conversion to the cross-β form is achieved. Because the α-helix is a rather flat helix, a chain which partially unwinds at the ends of a short helix does not have to change its general direction greatly in order to assume the cross-β form.

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Physical Properties of the Soluble RNA of *Escherichia coli*

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The soluble RNA of *Escherichia coli* has been prepared by a method ensuring that over 90% of the binding sites for amino acids are occupied. The homogeneity of the preparations has been studied in the ultracentrifuge and the weight-average value of S/D was found to be $4{\cdot}9 \times 10^{-7}$ cm^{-2} sec^2, giving a molecular weight of 24,500. Differences in the sedimentation coefficients of the preparations before and after removal of the amino acids from the binding sites have been observed. X-ray diffraction studies and measurements of hyperchromicity effects produced by heating show that these preparations have a double helical structure. A study of the effects of heating and rapid cooling on the hyperchromicity, molecular weight, homogeneity and the amino-acid acceptor activity shows that this activity does not depend on the intactness of the secondary structure.

1. Introduction

Since the discovery by Hoagland, Zamecnik & Stephenson (1957) that an intermediate step in the incorporation of amino acids into microsomal proteins in rat liver is the formation of a complex between the amino acids and a low molecular weight RNA, similar fractions of RNA, commonly called soluble RNA to distinguish them from the particulate RNA in ribosomes, have been found in other mammalian and bacterial cells (Berg & Ofengand 1958; Schweet, Bovard, Allen & Glassman, 1958). It has been shown that amino acids are activated by ATP and activating enzymes, specific for each amino acid, forming enzyme-bound aminoacyladenylate compounds (Hoagland, Keller & Zamecnik, 1956). The aminoacyl groups of these can then be transferred to the acceptor RNA, each being esterified on the 2^1 or 3^1 hydroxyl of the terminal adenosine of a specific RNA molecule (Zachau, Acs & Lipmann, 1958; Preiss, Berg, Ofengand, Bergmann & Dieckmann, 1959). In the case of rat liver soluble RNA the terminal nucleotide sequence, pCpCpA, is necessary for acceptor activity for the fifteen amino acids tested (Hecht, Stephenson & Zamecnik, 1958). The existence of soluble RNA molecules with specific acceptor activities for different amino acids has been clearly demonstrated by fractionation studies (Smith, Cordes & Schweet, 1959; Holley & Merrill, 1959; Brown, Brown & Gordon, 1959). On the basis of these properties, Hoagland, Zamecnik & Stephenson (1959) have postulated that molecules of soluble RNA act as adaptors in protein synthesis. The existence of such adaptor molecules was predicted earlier by Crick (1957) on theoretical grounds. It is assumed that soluble RNA molecules contain specific sequences of nucleotides, which determine the amino acids that attach to the terminal binding sites, and that these nucleotides hydrogen-bond with complementary sequences in RNA molecules in ribosomes. The sequence of nucleotides in the latter is then assumed to determine the sequence of the amino acids in the corresponding polypeptide chains. This hypothesis therefore

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predicts an interaction between soluble RNA molecules charged with amino acids and ribosomal RNA. The structure of soluble RNA charged with amino acids is then of some interest in this connection. The work described below is a study of the physical properties of soluble RNA isolated from *Escherichia coli* by a method ensuring that its binding sites are nearly all combined with amino acids.

2. Experimental

(a) *Preparation of RNA*

Escherichia coli strain B was grown in continuous culture in a 14 litre fermentor vessel at a cell density of 10^9/ml. in a medium containing 10 g glucose, 6 g Difco Yeast Extract, 11 g K_2HPO_4 and 8·5 g KH_2PO_4 per litre. After emerging from the culture vessel the bacterial cells were cooled to 2 to 3°C by passage through a cooling coil and harvested continuously in a refrigerated Sharples centrifuge. The harvested cells were then washed twice by centrifugation with 0·001 M-tris (2-amino-2-hydroxymethylpropane-1 : 3-diol) buffer, pH 7·4 + 0·01 M-$Mg(CH_3COO)_2$ (Tissières & Watson, 1958) and stored frozen at − 20°C overnight. Batches of 100 g wet weight were then treated as follows. After thawing, the cells were ground with 5 vol alumina in a vibratory ball mill at 3°C for 1 hr and the ground material suspended in 300 ml. of 0·001 M-tris buffer, pH 7·4 and 0·01 M-$Mg(CH_3COO)_2$ at 0°C. Immediately after the alumina was removed by centrifugation at 2000 ***g*** at 0°C for 10 min, 50 ml. of a solution containing 0·4 mg/ml. of each of the following amino acids was added: L-aspartic acid, L-alanine, L-arginine hydrochloride, L-cysteine, L-methionine, L-glutamic acid, L-*iso*leucine, glycine, L-histidine hydrochloride L-leucine, L-lysine hydrochloride, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan and L-valine. This was followed by 20 ml. of a solution containing 110 mg dipotassium ATP, 250 mg potassium phosphoenol pyruvate and 5 mg pyruvate kinase in 0·03 M-$Mg(CH_3COO)_2$ adjusted to pH 7·4. Without the addition of the amino acids, ATP and the ATP generating system, the soluble RNA subsequently isolated was not combined with amino acids. The suspension was then centrifuged at 34,000 ***g*** at 4°C to remove fragments of cell membrane. The supernatant was digested with 100 μg/ml. DNase for 15 min, then cooled to 3°C and centrifuged at 78,000 ***g*** for 5 hr. The clear supernatant above the pellet of ribosomes was removed and shaken with an equal quantity of aqueous 90% phenol (Gierer & Schramm, 1956; Kirby, 1956), prepared from redistilled phenol, for 1 hr at 20°C and then overnight at 3°C. The mixture was centrifuged for 1 hr at 1450 ***g*** at 0°C. The top layer was removed and replaced by an equal quantity of the tris-Mg buffer, mixed and centrifuged at 1450 ***g*** for 20 min. The two top layers were pooled and the phenol removed by 3 extractions with 50 ml. peroxide-free ether. Residual ether was removed by bubbling nitrogen through the solution at 0°C. The solution was then made 2% with respect to potassium acetate and the RNA precipitated by addition of 2·5 vols 96% ethanol. The precipitate was redissolved in 2% potassium acetate and reprecipitated in ethanol twice. The precipitate was dissolved in 20 ml. of 0·1 M-NaCl, the pH adjusted to 7·0 with 1·0 N-acetic acid, and the slight precipitate removed by centrifugation at 1,450 ***g*** for 20 min at 0°C. Solid NaCl was then added to make the solution 1 M and the temperature of the solution lowered to − 8°C and left at that temperature for 30 min. The precipitate that formed was removed by centrifugation at 34,000 ***g*** for 30 min and the supernatant dialysed for 8 hr against 1 M-NaCl at 3°C in dialysis tubing washed with 0·1% EDTA (ethylenediaminetetra-acetic acid) and hot distilled water. The dialysed RNA was then precipitated with 2·5 vols 96% ethanol, dissolved in 0·1 M-NaCl and reprecipitated in 2·5 vols ethanol and the precipitate finally dissolved in water and dialysed against water for 6 hr and then frozen-dried.

(b) *Ultracentrifuge measurements*

These were all obtained with the Spinco model E ultracentrifuge at 20 ± 0·1°C in 0·02 M-phosphate buffer, pH 6·8. For the sedimentation velocity experiments the ultraviolet absorption optics were used and the sedimentation boundaries recorded photographically. The Spinco Analytrol densitometer was used to convert the photographic records into graphs of RNA concentrations as a function of position in the cell.

The measurement of the ratio of sedimentation coefficient to diffusion constant (S/D) was made by the modification of the method of Archibald (1947) by Ehrenburg (1957) which uses the boundary condition at the meniscus in the centrifuge cell. A phaseplate was used in place of the diaphragm of the schlieren optics and a 77A Wratten filter was placed in the light filter holder. The cell used was a 12 mm synthetic boundary cell with a rubber valve. The soluble RNA was dialysed against the phosphate buffer for 12 hr at 0°C instead of water as in the last stage of the preparation. The first centrifuge run was made with 0·4 ml. of RNA solution in the cell and without any solvent in the cup of the boundary-forming cell. A series of schlieren photographs was taken beginning at a time when the intersection between the refractive index gradient curve and the meniscus image was clearly defined. A second run was made immediately after the first with the same volume of RNA solution in the same cell under identical conditions of acceleration time, speed of rotation, temperature, phaseplate angle, exposure times and intervals, but with equilibrated solvent from the dialysis in the cell cup which was layered over the RNA solution at the beginning of the run. The photographs were enlarged and the height (y) of the intersection of the refractive index gradient curve with the meniscus image above the base line, for each one taken during the first run, and the position of the meniscus in the cell were measured. The area (A) between the curve, the meniscus image and the base line for the first run and the area (A_s) between the curve and the base line for the second run were measured with a planimeter. Values of S/D for each corresponding pair of exposures were calculated from the equation $S/D = \frac{y}{w^2 r_m k_2 (A_s - A)}$ in which w is the angular velocity of the rotor, r_m is the radius of the meniscus and k_2 is the enlargement factor.

(c) *X-ray diffraction techniques*

A 10% aqueous solution of the soluble RNA was allowed to dry at room temperature on a glass slide. The resultant dried film was removed with a razor blade and mounted in slight tension with the plane of the film parallel to the X-ray beam in an X-ray camera. The specimen-to-film distance was 46 mm and the camera contained hydrogen at a known relative humidity (Langridge, Wilson, Hooper, Wilkins & Hamilton, 1960).

(d) *Other techniques*

The variation of ultraviolet absorption with temperature was observed with a Beckmann model DU spectrophotometer with coils in the walls of the cell compartment through which water was circulated from a thermostatically controlled water bath.

Amino-acid acceptor activity was measured by incubating samples of RNA from 0·1 to 1·0 mg with 10 μmoles dipotassium ATP, 100 μg of the dialysed supernatant from the sedimented ribosomes, 0·05 mg ^{14}C-labelled *Chlorella* protein hydrolysate, specific activity $1{\cdot}1 \times 10^7$ cts/min/mg, 30 μmoles of $MgCl_2$ and 200 μmoles tris buffer pH 7·4 in 1 ml. water at 37°C for 15 min. The reaction was stopped by cooling to 0°C and making the mixture 0·4 M-$HClO_4$ after addition of 5 mg bovine serum albumin. The resulting precipitate was washed successively with cold 0·4 M-$HClO_4$, cold 5:1 ethanol, 0·2 M-$HClO_4$ and ethanol. The washed precipitate was suspended in ethanol and filtered on to paper discs and the incorporated activity counted with an end-window geiger counter.

Contaminating polysaccharides were determined by the method described by Littauer & Eisenberg (1959) and deoxyribonucleotide impurities by the method of Burton (1956). Protein impurities were estimated by first removing the attached amino acids by incubation with 0·2 M-$NaHCO_3$, pH 10·0 for 2 hr and precipitation with 2·5 vols ethanol, digesting the precipitate with 6 N-HCl at 100°C for 8 hr and running the digest on a 2-dimensional paper chromatogram (Wellington, 1951) and staining with ninhydrin and comparing with standard spots of amino acids. Total phosphorus was estimated by the method of Allen (1940) after digestion according to Peacocke (1951).

3. Results

Three preparations of the sodium salt of soluble RNA made from *Escherichia coli* cells by the method described were found to have phosphorus contents of (1) 8·6%,

(2) 8·4%, (3) 8·4%. Preparation 2, which was used for most of the studies described here unless otherwise stated, contained 0·9% polysaccharide, less than 0·3% protein and 1·3% deoxyribonucleotides as impurities.

In the ultracentrifuge, all three preparations sedimented as a single boundary with sedimentation coefficients given in Table 1. Fig. 1(a) shows the variation of S/D with

TABLE 1

Sedimentation coefficients of soluble RNA

Prep. no.	Solvent	Preliminary treatment	Concentration	S
1	0·02 M-potassium phosphate pH 6·8	Freshly dissolved	0·003%	2·74
1	,,	Incubated 30 hr at 25°C	0·003%	3·42
2	,,	Freshly dissolved	0·003%	2·50
2	,,	Freshly dissolved	0·4%	2·56
2	,,	Incubated pH 10·0 at 30°C for 1 hr, and precipitated in ethanol	0·003%	3·75
3	,,	Freshly dissolved	0·003%	2·82
3	0·1 M-tris pH 7·4 + 0·01 M-magnesium acetate	Freshly dissolved	0·003%	2·89
3	,,	Incubated pH 10·0 at 30°C for 1 hr and precipitated in ethanol	0·003%	3·93

time of centrifugation measured from the instant when the rotor attained equilibrium at the selected speed, 17,250 rev/min for preparation 2. Ehrenberg (1957) has shown theoretically that, in the case of mixtures of components with different values of S/D, the apparent value of S/D measured in this way will decrease from the weight-average at zero time of centrifugation to the lowest value of S/D with increasing time of centrifugation. Only in the case of a solute that is homogeneous with respect to S/D and for which this ratio is independent of the concentration of the solute, will the measured value of S/D remain constant during centrifugation. From Fig. 1(a) it can be seen that the decrease in the value of S/D with time of centrifugation for the soluble RNA preparation was very small during the period of centrifugation when y could be accurately measured. The value of S/D at zero time of centrifugation obtained by extrapolation from the measured values was $4{\cdot}9 \pm 0{\cdot}2 \times 10^{-7}$ cm^{-2} sec^2. Using the value of 0·51 obtained by Tissières (1960) for the partial specific volume of the soluble RNA of *Escherichia coli*, substitution of this value of S/D in the Svedberg equation (Svedberg & Pederson, 1940) gives a value of 24,500 ± 1,000 for the molecular weight of preparation 2.

Fig. 1(b) shows the variation of S/D with centrifugation time of the same preparation after heating to 90°C for 1 hr in 0·02 M-potassium phosphate buffer pH 6·8 at a

concentration of 8·9 mg/ml., and then cooling rapidly to 20°C within one second. The slope of this curve indicates that the preparation treated in this manner was very inhomogeneous and the value of S/D obtained by extrapolation of the measured points to zero time of centrifugation was $1{\cdot}1 \times 10^{-6}$ cm $^{-2}$ sec^2, which gives a weight average molecular weight of 57,000 $\pm$ 2,000.

Table 1 shows that, when freshly dissolved, the sedimentation coefficients of the three preparations were between 2·55 and 2·95. After incubation at 30°C at pH 10·0 for 1 hr, a procedure which has been shown by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) to remove bound amino acids from rat liver soluble RNA, the sedimentation coefficients increased to between 3·75 and 4·05. Incubation at 25°C at pH 6·8 for 30 hr caused the sedimentation coefficient of preparation 1 to rise from 2·74 to 3·42.

Fig. 2 shows the results of tests of the ability of the soluble RNA preparation 2 to

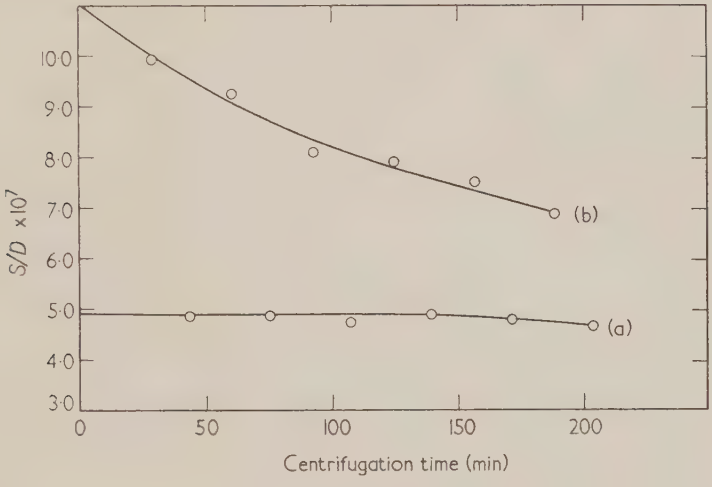

Fig. 1.

(a) The variation of values of S/D, calculated from the schlieren photographs, with time of centrifugation at 17,250 rev/min for preparation 2 at a concentration of 8·9 mg/ml.
(b) The variation of S/D with time of centrifugation at 12,590 rev/min for preparation 2, at a concentration of 8·9 mg/ml., after heating to 90°C for 1 hr in 0·02 M-potassium phosphate buffer, pH 6·8 and cooling rapidly to 20°C.

bind the mixture of ^{14}C-labelled amino acids in a *Chlorella* protein hydrolysate after the following treatments. Curve 2(a) was obtained after the bound non-radioactive amino acids had been removed from the soluble RNA by incubation with 0·2 M-$NaHCO_3$ buffer, pH 10·0 at 30°C for 1 hr and the RNA precipitated with 2·5 vols ethanol and redissolved in 0·2 M-tris buffer, pH 7·4. Curve 2(b) was obtained after 20 mg of the soluble RNA freed of bound amino acids had been incubated with 4 μmoles of sodium metaperiodate in 0·1 M-succinate buffer, pH 5·6 at 25°C for 30 min, then precipitated with 2·5 vols ethanol, redissolved in 0·2 M-tris buffer, pH 7·4 and dialysed 6 hr against 2 litres of this buffer. Preiss *et al.* (1959) have shown that this treatment oxidizes the ribose component of the terminal nucleotide, inactivating the amino-acid binding sites unless these are protected by bound amino acids. Curve 2(c) shows the effect on the amino-acid acceptor ability when the periodate treatment was applied to the original preparation containing bound amino acids. The small difference between the slopes of 2(a) and 2(c) shows that over 90% of the binding

sites with respect to the amino acids found in a *Chlorella* protein hydrolysate must have been filled with amino acids. Curve 2(d) was obtained after heating the soluble RNA to 90°C for 1 hr at a concentration of 9·3 mg/ml. in 0·02 M-potassium phosphate buffer, pH 6·8, cooling rapidly to 20°C and then removing the remaining bound amino acids as in the case of 2(a). The ability to bind amino acids was not appreciably affected by this treatment.

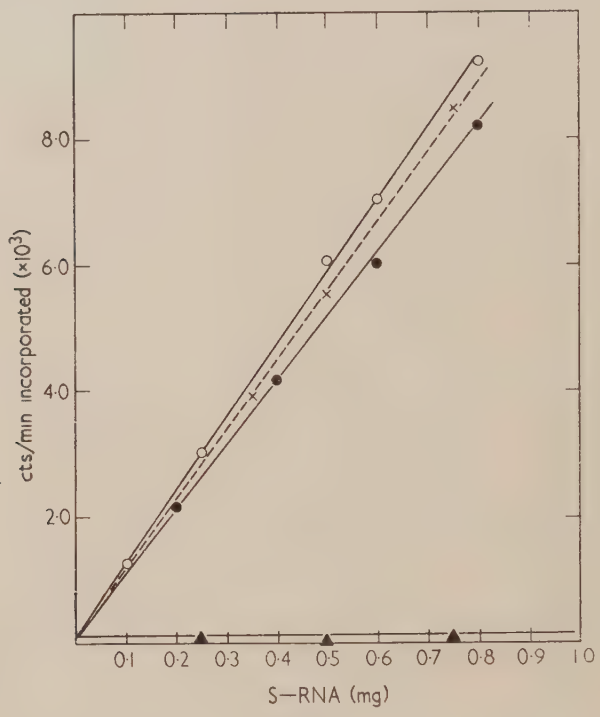

FIG. 2.

Incorporation of ^{14}C-labelled *Chlorella* protein hydrolysate, specific activity $1{\cdot}1 \times 10^7$ cts/min/mg, by S-RNA after the following treatments:

(a) —○——○——○—, incubated at pH 10·0, 30°C, for 1 hr.
(b) —▲——▲——▲—, incubated at pH 10·0, 30°C, for 1 hr and then with sodium metaperiodate at pH 5·6, 25°C, for 30 min.
(c) —●——●——●—, incubated with sodium metaperiodate at pH 5·6, 25°C, for 30 min.
(d) - - × - - × - - × - -, heated to 90°C for 1 hr at a concentration of 9·3 mg/ml. at pH 6·8, cooling rapidly to 20°C and incubated at pH 10·0, 30°C, for 1 hr.

Fig. 3 shows the variation of optical density at 260 mμ of the soluble RNA on heating to temperatures from 25°C to 90°C at a concentration of 0·004% wt/vol in 0·02 M-potassium phosphate buffer pH 6·8 during which the optical density increases by 25%. On cooling slowly over a period of 4 hr, the subsequent cooling curve (not shown) followed the heating curve within 2% which indicates that the hyperchromic effect is reversible at this low concentration. On heating another sample to 90°C for 1 hr in 0·02 M-potassium phosphate buffer at a concentration of 9·3 mg/ml. and then cooling rapidly to 20°C within one second, the specific extinction coefficient at 260 mμ $E^{1\%}_{1cm}$ was found to have increased by 16% from 219·0 to 254·0. On repeating this treatment on a sample from which the bound amino acids had been previously removed by incubation at 30°C for 1 hr at pH 10·0, the specific extinction coefficient rose from 226·0 to 251·0.

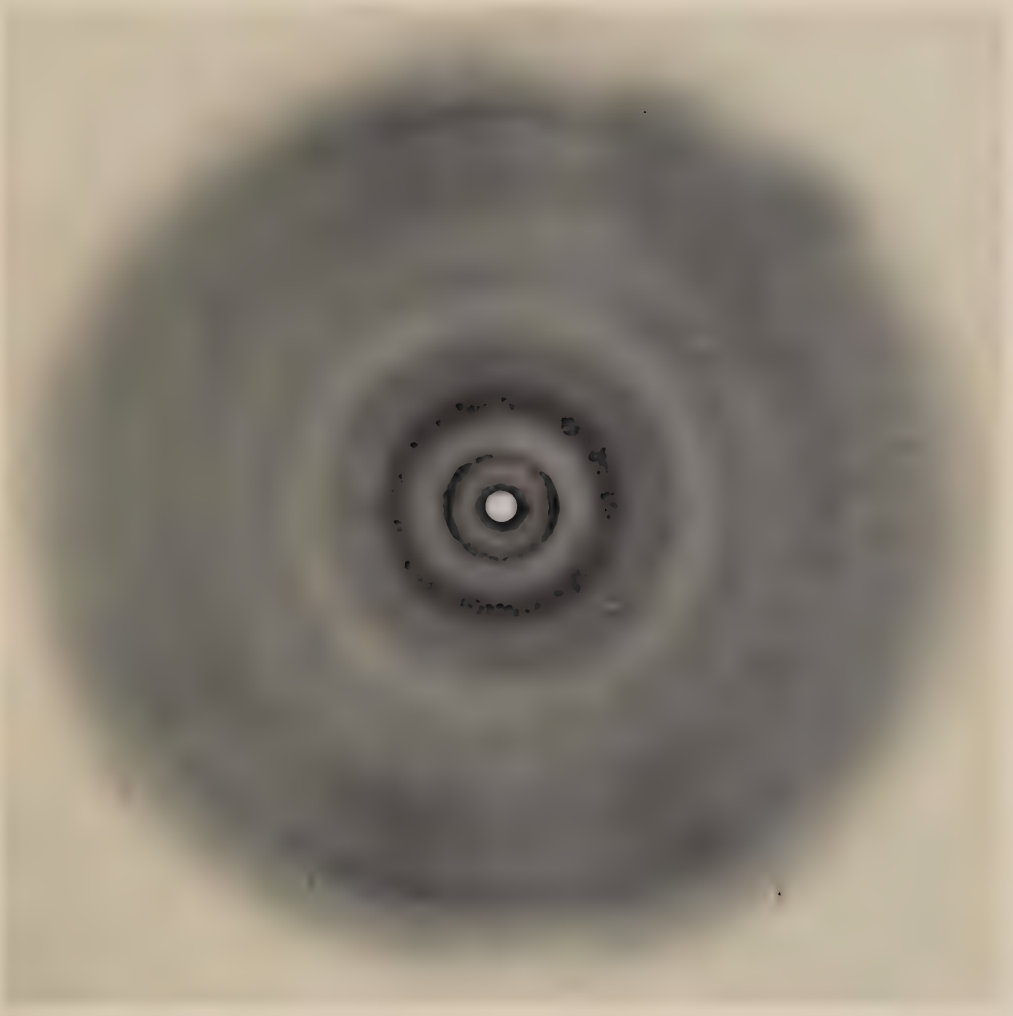

PLATE I. X-ray diffraction pattern of film of soluble RNA at 92% relative humidity.

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Plate I shows an X-ray diffraction pattern of the soluble RNA at 92% relative humidity. The pattern contains six prominent diffraction haloes: 22·7 Å, sharp, strong and equatorially oriented; 11·7 Å, strong and diffuse; 7·5 Å, diffuse; 5·6 Å, diffuse; 4·0 Å, diffuse; and 3·3 Å, meridionally oriented. There is also a faint reflection at 3·8 Å on the equator which may be due to an impurity or to a higher order equatorial spacing. The diffraction pattern changes reversibly with relative humidity. When the soluble RNA is in the dry state, the pattern vanishes. Between 75% and 98% relative humidity, the equatorial spacing expands from 19·3 Å to 25·1 Å.

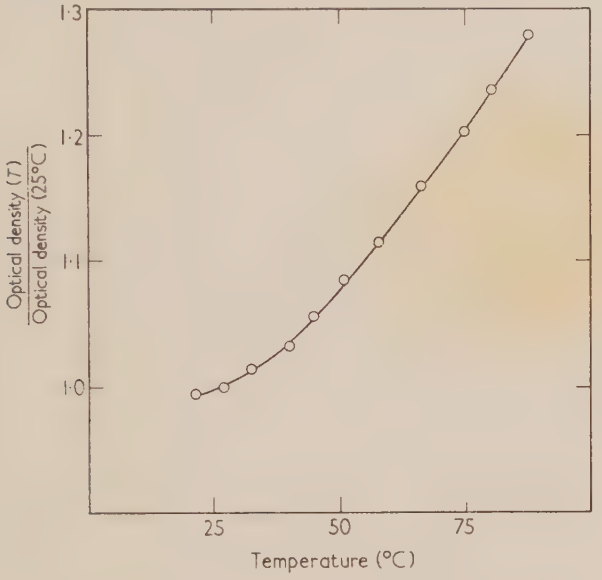

FIG. 3.

The variation of optical density at 260 mμ of soluble RNA with temperature at a concentration of 0·004% wt/vol in 0·02 M-potassium phosphate buffer.

4. Discussion

The effect of the metaperiodate treatment on the binding of ^{14}C-labelled amino acids to the soluble RNA shows that the preparation described here was one in which nearly all the binding sites were filled with amino acids. The ultracentrifuge data show that the preparation was homogeneous with respect to S/D and therefore with respect to molecular weight. The variation of S/D with centrifugation time of the heated material indicates that the ultracentrifuge technique used was sensitive to inhomogeneity in the molecular weight range of the soluble RNA. The molecular weight of the preparation studied was 24,500 $\pm$ 1,000, considerably higher than that previously obtained by separate measurements of S, D and the intrinsic viscosity, η, (Brown *et al.*, 1959) on preparations made by a slightly different method. It is clear from the data in Table 1 that preparations of soluble RNA charged with amino acids as described here are unstable with regard to their sedimentation coefficients. Separate measurements of S, D and η, made necessarily under different observational conditions, are not therefore suitable for the calculation of the molecular weight of this material. The measurement of the ratio S/D in one experiment as described here overcomes this difficulty. The molecular weight thereby obtained may be considered more reliable for this type of RNA preparation than that made previously. Tissières

(1960) has recently described the physical properties of the soluble RNA of *Escherichia coli* made by a method which, in our experience, gives a product with most of the binding sites unoccupied by amino acids. It was found that the molecular weight values for this material, obtained from separate measurements of S, D and η, were 25,500 $\pm$ 2,500 and 26,400 $\pm$ 4,000. The sedimentation coefficient did not vary with time and had an average value of 4·0 s. Thus, within the limits of experimental error, the molecular weights of the two kinds of preparations are similar, but the sedimentation coefficients differ considerably. This, together with the observed increase in the sedimentation coefficient of our preparations after removal of the amino acids at pH 10·0, suggests that the soluble RNA/amino acid complexes isolated direct from *Escherichia coli* cells have a more extended structure with a lower sedimentation coefficient than the soluble RNA alone. Accordingly the variation with time of the sedimentation coefficient at 20°C of the soluble RNA combined with amino acids might be due to the spontaneous release of amino acids from the complexes. It is difficult to visualize how the attachment of an amino acid to the end of a soluble RNA chain could produce such a large change in the folding of the chain implied by the difference in the sedimentation coefficients. It may therefore be that the method of preparation used maintains an unstable state of the molecule which exists in the cell. It is noteworthy in this connection that during the preparation the soluble RNA is effectively incubated at 4°C with ATP, amino acids and all the components from the disrupted cells.

It has been suggested by Doty, Boedtker, Fresco, Haselkorn & Litt (1959) that the hyperchromic effect observed in a nucleic acid on heating may be used as a measure of the number of internucleotide hydrogen bonds and of the fraction of the molecule in a helical configuration. If it is assumed that a 40% rise in optical density at 260 mμ is proportional to the breakage of 2·0 to 2·5 hydrogen bonds per nucleotide, as in thymus DNA, then a rise of 28% in optical density on heating the soluble RNA in dilute solution, as observed here, suggests a breakage of at least 1·5 hydrogen bonds per nucleotide. Thus, if all the hydrogen bonds in the RNA have been broken on heating to 85°C, this would indicate that there are at least 1·5 internucleotide hydrogen bonds intact in the nucleic acid solution at room temperature. The most reasonable structure involving a large number of internucleotide hydrogen bonds would be a double helix, something like DNA. Since the rise in optical density on heating is gradual and reversible at low concentrations, unlike that in native thymus DNA, which is abrupt and only partly reversible, it is likely that the hydrogen bonds are not formed between two polynucleotide chains with complementary nucleotide sequences, as in DNA, but in a more irregular fashion.

The diffraction pattern of a film of the RNA bears a strong resemblance to the diffraction pattern given by native sodium DNA at high humidity in the B-configuration (Wilkins, Stokes & Wilson, 1953; see Fig. 5, Wilkins, 1956). The partly oriented equatorial spacing at 22·7 Å corresponds to the equatorial and first layer-line reflections of an oriented DNA B pattern; the 11·7 Å ring corresponds to the strong inner part of the second layer line, the 7·5 Å ring to the inner part of the third layer line, the 5·6 Å ring to parts of the fifth, third, second and zero layer lines, the 3·8 Å ring mainly to the eighth layer line, and the 3·3 Å meridional spacing to the tenth. The innermost ring and the 3·3 Å meridional reflection are less intense in the RNA pattern than in that of DNA. This interpretation of the diffraction pattern is identical to that given for the diffraction pattern of the microsomal RNA from *Escherichia coli* (Zubay & Wilkins, 1960). The orientation observed here in the equatorial 22·7 Å and

meridional 3·3 Å spacing strongly confirms the assignment for these spacings and shows a real difference between the RNA patterns studied by us and those described by others (Rich & Watson, 1954). At present, the significance of these differences is not clear. The single meridional reflection and the closer resemblance between this pattern and the *B* structure of DNA makes it considerably clearer that the RNA studied here is in a double helix structure like DNA. The pattern is not as well oriented as DNA; but this is not surprising in view of the low molecular weight of the RNA, 24,500, which makes it very difficult to orient by the usual procedure of drawing a fibre. The orientation observed in the film indicates that the long axis of the double helix prefers to lie in the plane of the film, and the extreme sharpness of the equatorial reflection indicates a regularity in spacing between molecules only paralleled in nucleic acids by the best samples of DNA which have been studied by X-ray diffraction methods.

It appears, then, that soluble RNA has a double helical configuration, but not necessarily with regular base pairing since the hyperchromicity data suggest that less than three-fourths as many hydrogen bonds are formed per nucleotide in soluble RNA as in native thymus DNA. It has already been argued (Zubay & Wilkins, 1960) that the strongest internucleotide hydrogen bonds are formed between 6-keto and 6-amino groups and that this may account for the ratios of bases in most RNAs (Elson & Chargaff, 1953). It is possible to form pairs of hydrogen bonds between 6-keto and 6-amino groups if the opposing bases are attached to two antiparallel polynucleotide chains as in DNA. In the case of the soluble RNA this makes two structures possible. Two RNA chains might interact together to form a double helix or, alternatively, one RNA molecule might bend near the middle and double back on itself to form a double helical structure where the two ends of the chain come in close contact. The second choice is strongly supported by the effect of heating to 90°C (where all the hydrogen bonds will be broken) and subsequent rapid cooling on the molecular weight and the homogeneity of the soluble RNA in concentrated solution. The fact that the weight average molecular weight nearly doubles after this treatment suggests that the hydrogen bonds in the original material were formed between nucleotides in the same chain and that the hydrogen bonds formed on cooling tended to be between nucleotides on different chains. The reversibility of the hyperchromic effect in dilute solution indicates that the original hydrogen bonds may be re-formed under this condition, where there is little chance of forming intermolecular hydrogen bonds. The second choice is also supported by end-group studies (Brown & Kosinski, unpublished) which indicate that there are 83 nucleotides per polynucleotide chain, giving a molecular weight only about 15% greater than that obtained by the ultracentrifuge measurements described above.

Thus the most probable structure seems to be a single chain of 70 to 80 nucleotides folded back on itself in the form of a double helix. This raises the possibility that this structure may be necessary for the biological activity of the soluble RNA. This activity has been shown in other soluble RNAs to be the specific binding of amino acids to one end of the RNA chains (the amino-acid acceptor ability) and the subsequent transfer into peptide linkage in ribosomes (the transfer ability). The effect of heating and rapid cooling of concentrated solutions shows that the amino-acid acceptor ability is not dependent on the intactness of the secondary structure. This activity is almost unaffected by this treatment whereas the changes in molecular weight, homogeneity and specific extinction coefficient show that the secondary structure is drastically altered. It therefore seems unlikely that the specific binding of the amino

acids on one end of the chain requires the co-operation of the other end. This suggests that the nucleotide sequences determining the specificity of the amino-acid binding sites of the soluble RNA are adjacent to those sites. The possibility that the secondary structure is necessary for the transfer properties of the soluble RNA requires further investigation.

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Infection of Protoplasts of *Escherichia coli* by Subviral Particles of Bacteriophage ϕX174†

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Infection of protoplasts of various strains of *Escherichia coli* may be obtained with purified DNA preparations from bacteriophage ϕX174 made by two distinctly different methods. Infection of such protoplasts can also be obtained with nucleoprotein particles made from ϕX174 by heat treatment. None of the particles, infective to protoplasts, are infective to whole cells and all are infective to protoplasts of strains of bacteria usually resistant to whole ϕX174 virus.

The protoplast-infective agents are in all cases destroyed by enzymatic treatment with DNase. The infective DNA is not inactivated by treatment with enzymatic amounts of trypsin but is by treatment with stoichiometric amounts, suggesting the formation of an inactive DNA-trypsin complex.

Studies of the properties of protoplast infection indicate certain general characteristics which distinguish it from the more familiar virus infection of whole bacterial cells.

1. Introduction

The use of protoplasts for the study of microbiological phenomena is becoming increasingly widespread. Investigations conducted more or less simultaneously by Spizizen (1957) and Fraser, Mahler, Shug & Thomas (1957) demonstrated that protoplasts could be infected by subviral particles of the bacteriophage T2. Fraser has discussed the general characteristics of such infections.

Bacteriophage ϕX174 has been characterized by Sinsheimer (1959*a*) as a minute particle containing a single molecule of DNA. This DNA molecule has been characterized physically and chemically (Sinsheimer, 1959*b*) as a small single-stranded polynucleotide of some 5,500 nucleotides. For these reasons, attempts have been made to obtain, from ϕX174, subviral particles with the ability to infect protoplasts. Such infective particles have been obtained by three different methods. These particles are of two types. One type is known from its behavior during sedimentation and from its density to have associated with it a large part of the original viral protein. The other type is the free nucleic acid of the virus. Evidence for the fact that this latter particle is the free DNA rests to a large extent on the physical-chemical characterization of the molecule as reported by Sinsheimer.

2. Materials and Methods

Stock solutions

All stocks including nutrient media are made up using either deionized water or double distilled water.

Tris buffers: Sigma 121 grade (Sigma Chemical Co., St. Louis, Mo.) is used in stock solutions of tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer.

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Lysozyme: Worthington Biochemical Co. crystalline egg white lysozyme is made up weekly to a concentration of 2 mg/ml. in water, and stored in the cold.

EDTA: a 4% solution of EDTA (ethylenediaminetetra-acetic acid, Eastman Organic Chemicals) dissolved in water.

Modified 3XD broth: this is the casamino acid-glycerol broth of Fraser & Jerrel (1953) but contains only 0·9 g KH_2PO_4 and 2·1 g Na_2HPO_4 per liter.

Nutrient broth: the nutrient broth in which protoplasts are incubated is a modification of that used by Lederberg & St. Clair (1958) for the growth of protoplasts and L-forms of *E. coli*. It contains 10 g Casamino Acids (Difco), 10 g Nutrient Broth (Difco—a mixture of beef extract and peptones, 3 to 5), 1 g glucose, 100 g sucrose, per liter of water. After autoclaving, 10 ml. of a sterile 10% solution of $MgSO_4$ (anhydrous) are added.

Assay Procedure

The agar layer method (Adams, 1950) is used. Top agar for plating infected protoplasts is the nutrient broth (above) plus 1% agar. After the top agar (2·5 ml.) is pipetted into the plating tubes, 0·15 ml. BSA (Armour and Co. 30% sterile solution bovine serum albumin) is added per tube plus 0·2 ml. plating bacteria. The top agar for the assay of mature virus contains 1 g Tryptone, 0·5 g NaCl, and 0·8 g agar per 100 ml. water.

Bottom agar, for both protoplasts and virus, is 10 g Tryptone, 2·5 g NaCl, 2·5 g KCl, 10 g agar, 6 ml. 1 N-NaOH per liter of water. After autoclaving 1 ml. sterile 1 M-$CaCl_2$ is added.

Plating bacteria: E. coli C are grown, with aeration, to log phase at 37°C in "adsorption medium" (10 g Tryptone, 5 g KCl, 5×10^{-4} M-$CaCl_2$ per liter of water). 0·2 ml. plating bacteria are used per plate and plaques can be counted after 3 to 4 hr incubation at 37°C.

Protoplasts: during the development of this work the following strains of *E. coli* have been used to make protoplasts: C(BTCC no. 122), C/ϕX (isolated in this laboratory from C), B (Luria), and K12 nonlysogenic (obtained from Dr. Jean Weigle). Protoplasts of all of these strains can be infected by subviral particles with about the same efficiency but vary somewhat in ease of handling. All of the strains, after conversion to protoplasts by our method, exhibit, to some degree, the ability to reform cell walls and divide. As a consequence, the term protoplast is applied with some reservation. The particles are, however, spherical in shape, and "shock" when diluted 1 : 20 into water. With the exception of *E. coli* C, these strains are completely resistant to infection with whole virus ϕX174 when plated by our usual procedure. Also, incubation of either K12 cells or K12 protoplasts with whole virus under the same conditions as those used for infection with DNA produces no evidence of infection of even a small percentage of the cells or protoplasts.

To obtain protoplasts, cells of the desired strain† are inoculated, from either a slant or a saturated culture, into modified 3XD broth and allowed to grow at 37°C with aeration to 5×10^8/ml. (as determined in the Petroff-Hausser counting chamber). The cells are subcultured with a dilution of 1 : 25 into prewarmed medium and allowed to grow again to a concentration of 5×10^8/ml. (The concentration limits here are approximately 2 to 6×10^8/ml.)

Twenty ml. cells are then centrifuged at room temperature, 1,000 *g* for 10 min. The pellet is taken up in 0·35 ml. 0·5 M-sucrose; 0·1 ml. 0·25 M-Sigma 121 buffer pH 8·1 and 0·01 ml. lysozyme stock are added, then 0·02 ml. 4% EDTA. (Note that the cells are neither washed nor chilled.) Formation of protoplasts is complete (99·9%) within 5 to 10 min at room temperature. After 10 min the protoplasts are diluted fivefold into nutrient broth containing 2% BSA. A further fourfold dilution is made after 3 to 5 min into nutrient broth to reduce the concentration of BSA. This is the "protoplast stock."

Subviral Particles

In making each of the subviral particles a purified preparation of ϕX174 (Sinsheimer 1959*a*) is used.‡

†*E. coli* B (Luria) cannot be converted to protoplasts as easily as the other strains. The method of Fraser *et al.* (1957) has been used successfully for this strain. These protoplasts are more stable and will survive centrifugation at 1,000 *g* for 10 min at room temperature, without loss of activity.

‡A fourth method for obtaining subviral particles, treatment with 8 M-urea (Fraser *et al.*, 1957) has also been used. This method produces a mixture of protein containing particles and free DNA but in general the particles do not appear to be as infective as the others described.

I. Heated particle† (*HTϕX*): purified ϕX (at a concentration of 10^{15}/ml.) is diluted 1/100 into 0·1 M-tris buffer pH 7·5. It is then heated at 70°C for 5 min and cooled. Samples treated in this manner show a loss of infectivity (as assayed by normal plating procedure) of between 10^6 and 10^8/ml. From electron micrographs of this particle (Hall, Maclean & Tessman, 1959) it appears that, on heating, the virus breaks open and that the DNA, possibly with some protein, is extruded from the protein coat. HTϕX has a density in rubidium chloride of 1·42 g/cm^3 (0·02 g/cm^3 greater than the whole virus). Its sedimentation coefficient in 0·1 M-tris pH 8·0 is 47 S, less than that of either the whole virus (114 S) or the protein "coat" (70 S), a result which would be expected for a particle of the type seen in the micrographs. Upon treatment with DNase the sedimentation coefficient of the residual protein particle rises to 70 S. Also present in the HTϕX preparation is a small amount of material which is probably free DNA. This material may be separated from the HTϕX particle by banding in a cesium chloride density gradient (*vide infra*); both the DNA and HTϕX particle are biologically active when tested with protoplasts.

II. Phenol-prepared DNA: the preparation of this particle (the free DNA) is described and the molecule is characterized as the free DNA by Sinsheimer (1959*b*).

III. Calcium particle (*CaϕX-DNA*): a third method for obtaining a biologically active subviral particle is the following: purified ϕX is diluted tenfold (from a stock of 10^{14} to 10^{15} virus/ml.) into cold 4 M-$CaCl_2$ (analytical reagent grade recrystallized once from 95% ethanol and dissolved in deionized water), allowed to stand at room temperature for 30 min and stored in the cold for another 90 min. A heavy precipitate forms which contains almost all of the virus. This is centrifuged in the cold for 30 min (12,000 ***g*** in the Servall SS 1 centrifuge). The supernatant‡ is discarded. The pellet is resuspended in 0·1 M-tris buffer pH 7·5 to 8·0, centrifuged and the supernatant retained. This supernatant contains from 30 to 50% of the original viral DNA. Repeated extractions of the pellet, which presumably contains the rest of the DNA, are largely unsuccessful.

The ultraviolet absorption spectrum of this calcium-prepared DNA is the same as the spectrum of the DNA from the phenol treatment with a minimum at 230 mμ and a maximum at 257 mμ. Its sedimentation coefficient is that of phenol-prepared DNA (23 S). In the cesium chloride density gradient it bands at a density of 1·72 g/cm^3 as does phenol-prepared DNA. On the basis of these criteria the CaϕX-DNA is believed to be the same molecule as that prepared by the phenol method. Data obtained using either of these particles are reported as data resulting from the infection of protoplasts by free ϕX-DNA.

General Method for Infecting Protoplasts with Subviral Particles of $\phi X174$

Cells are grown and converted to protoplasts as described above. The infecting particle, which is stored in 0·1 M-KCl, 0·001 M-tris buffer, pH 7·5 (under these conditions infectivity is retained for weeks if the sample is kept in the cold), is diluted at least tenfold into 0·01 M-tris pH 8·0 to 8·3. To a volume of infecting particles is added an equal volume of "protoplast stock." The mixture is incubated at 37°C for 10 to 15 min, after which it is diluted fivefold into nutrient broth. After another 90 min incubation the sample is titered for mature ϕX phage. If infective centers are to be assayed the sample is diluted and plated after the 10 min adsorption period. Dilutions for infective centers are made through nutrient broth plus 2% BSA and plating is performed as described above.

Experiments have shown that, while 2% BSA is necessary for the initial stabilization of the protoplasts, the BSA concentration during incubation of the infected protoplasts in liquid medium must be below 0·2% for maximum final phage yield. During incubation of the protoplasts on agar plates, however, BSA at a concentration of from 1 to 2% is used.

3. Experiments and Results

Banding of the infective particles in the cesium chloride density gradient: each of the three particles has been banded by cesium chloride density gradient centrifugation (Meselson, Stahl & Vinograd, 1957). After fractionation by drop collection from the

† This method for obtaining a subviral particle was suggested to us by Dr. Irwin Tessman.

‡ This first supernatant has ultraviolet absorption with a maximum at 270 mμ and apparently contains a small amount of viral protein.

sample tube the fractions were assayed for biological infectivity using protoplasts and were also assayed for ultraviolet absorption in the Beckman DK-2 Spectrophotometer. In each case the biological activity was found in the fractions corresponding to those with the u.v. absorption spectrum of the particle. The amount of infectivity corresponds reasonably directly to the particle concentration as calculated from the u.v. absorption. Examples of such experiments are shown in Figs. 1 and 2. One of these experiments (CaϕX-DNA, Fig. 2) was done in a manner to permit compensation for the nonlinearity of assay (*vide infra*); the other was not. *Particles banded in this manner and assayed by the usual plating procedure for viable phage produce no plaques.*

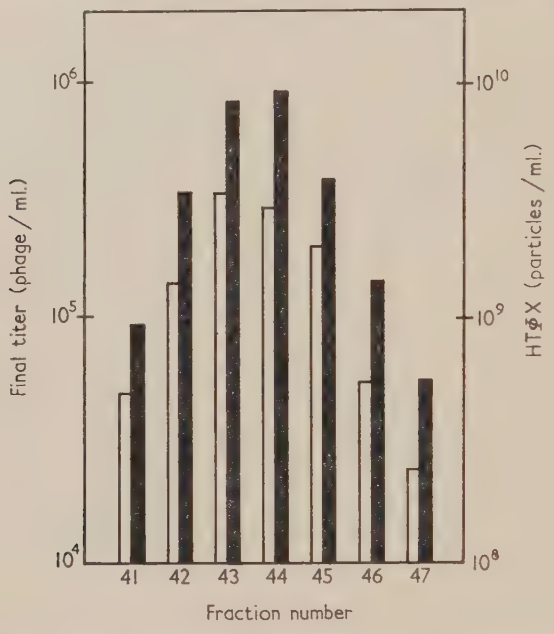

FIG. 1. Infectivity and particle concentration of samples from a cesium chloride density gradient band of HTϕX particles. Mean density of the band is 1·42 g/cm^3. Biological activity is represented by open bars; particle concentration, as calculated from u.v. absorption, by solid bars.

Growth curves: the multiplication of phage in infected protoplasts can be followed in a manner similar to that used for virus infected whole cells. Figs 3 and 4 show the results of such experiments using *E. coli* C protoplasts and *E. coli* K 12 protoplasts, respectively.

Each growth curve consists of two assays. One is made in a manner which measures total infective centres (infected protoplasts† plus free phage). This assay indicates the kinetics of "adsorption" and infection as well as the "latent period." The other curve is made by plating with an initial 1/20 dilution through water to "shock" any infected protoplasts in order to assay only mature phage particles. At later times of incubation, the latter assay is found to indicate 3 to 5 times more virus than the former. This is not a plating artifact since "shocked" samples plated in the same manner as infective centers (on BSA plates) give the same titer as those plated by the usual method. The result is interpreted to mean that protoplasts do not lyse in

†We do not know what fraction of the infected protoplasts survive and form infective centers on the agar plates. Characteristics of such growth on agar are discussed by Lederberg & St. Clair (1958).

the manner ordinarily observed with whole infected cells (due possibly to the presence of sucrose in the nutrient medium), and that a portion of the virus produced by the protoplasts is not released from them unless they are shocked into water.

Since the adsorption of DNA by protoplasts occurs in the presence of nutrient, events are measured from the time of mixing. The "zero time" infective centers simply represent those infections occurring between the time of mixing the DNA and protoplasts and the time the first sample is plated, i.e. within about the first minute. The mature phage curve (curve B of Figs. 3 and 4) is extrapolated back from the time of the first appearance of mature phage.

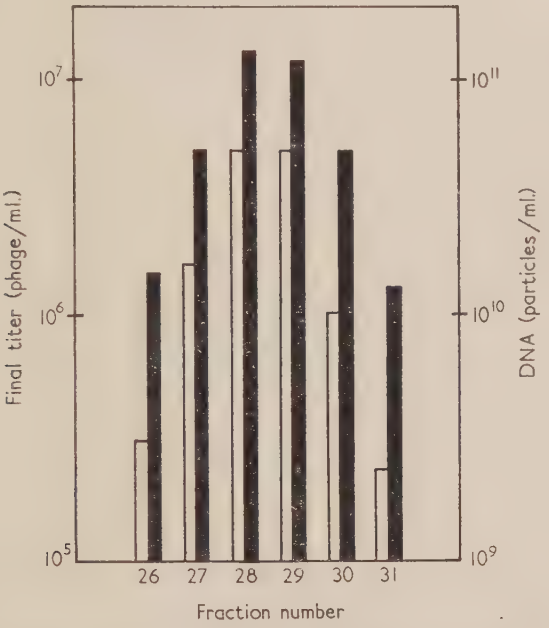

FIG. 2. Infectivity and particle concentration of samples from a cesium chloride density gradient band of CaϕX-DNA. Mean density of the band is 1·72 g/cm^3. Biological activity is shown by open bars; particle concentration, calculated from the u.v. absorption, by solid bars.

There is an obvious difference between the two growth curves at later times of incubation. The curve from the K12 experiment resembles more closely the shape of the growth curve of virus in whole cells, while the *E. coli* C curve continues to rise. *E. coli* C is a normal host for ϕX while K12 is not; also, protoplasts made by our method seem to show some capability of resynthesizing cell wall. These facts, coupled with the observation that the infected tube of C protoplasts remains clear while the control (uninfected protoplasts) is found to contain a high concentration of whole cells after incubation, lead to the conclusion that residual C cells or protoplasts which have regrown a portion of their cell wall are infected by the phage initially produced by the infected protoplasts, resulting in the continued production of phage. For this reason it is preferable in these experiments to use protoplasts of an organism which is resistant to mature ϕX174.

Dilution experiments: if successive dilutions of DNA are used to infect a constant number of protoplasts, activity curves of the general shape of those shown in Fig. 5 result. At high concentrations of DNA there is an apparent saturation of protoplasts susceptible to infection. At lower concentrations of infecting particle a linear

relationship (plotted on log-log coordinates) is observed between phage yield and infecting particle concentration, while at still lower concentrations a higher efficiency of infection per DNA particle is observed. If the "saturation level" in the assay for infective centers (curve A, Fig. 5) is taken as an indication of the number of protoplasts capable of being infected, rather than an indication of some sort of interference

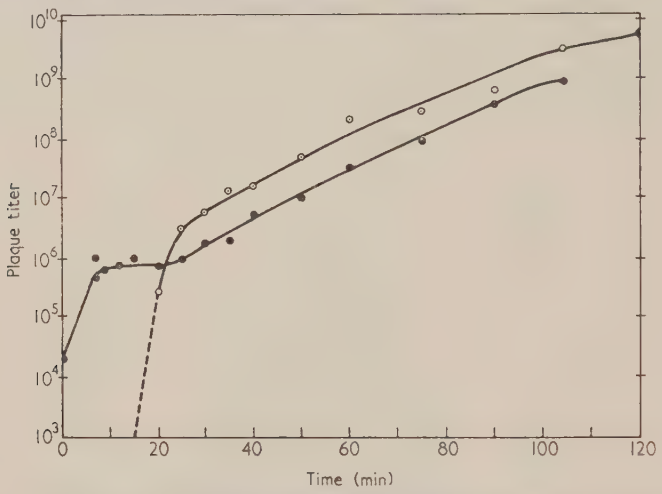

FIG. 3. Growth curve of ϕX174 using protoplasts of *E. coli* C infected by CaϕX-DNA. Curve A (●——●) indicates infective centers (infected protoplasts† plus free phage), curve B (⊙——⊙) mature phage.

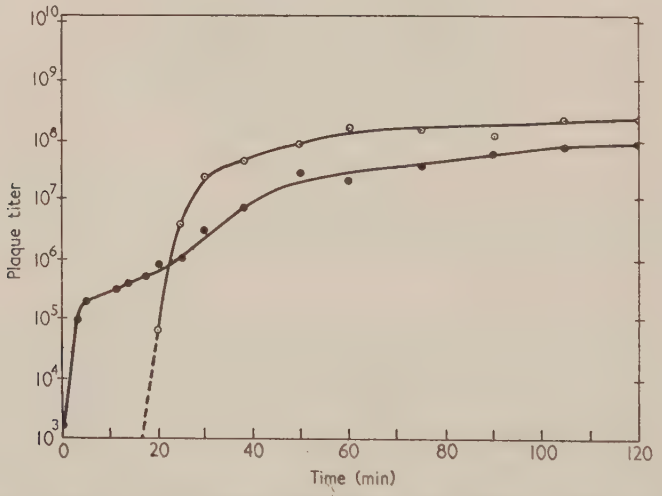

FIG. 4. Growth curve of ϕX174 using protoplasts of *E. coli* K 12 infected by CaϕX-DNA Curve A (●——●) indicates infective centers (infected protoplasts† plus free phage), curve B (⊙——⊙) mature phage.

phenomenon between DNA molecules which limits infection, the second break in the curve occurs at a concentration of infecting particles such that the multiplicity of DNA per susceptible protoplast is between 1 and 3. These data suggest that about 0·1% of the protoplasts can be infected by DNA, while a small fraction of these are more easily infected, and give rise to a greater efficiency at low DNA concentration.

† We do not know what fraction of the infected protoplasts survive and form infective centers on the agar plates. Characteristics of such growth on agar are discussed by Lederberg & St. Clair (1958).

At the lowest concentration represented in Fig. 5 the number of infective centers obtained is equivalent to 3% of the DNA particles (the number of DNA particles is calculated from the u.v. absorption). The final phage yield at this concentration contains 5·5 times as much ϕX-DNA in mature phage particles as was initially

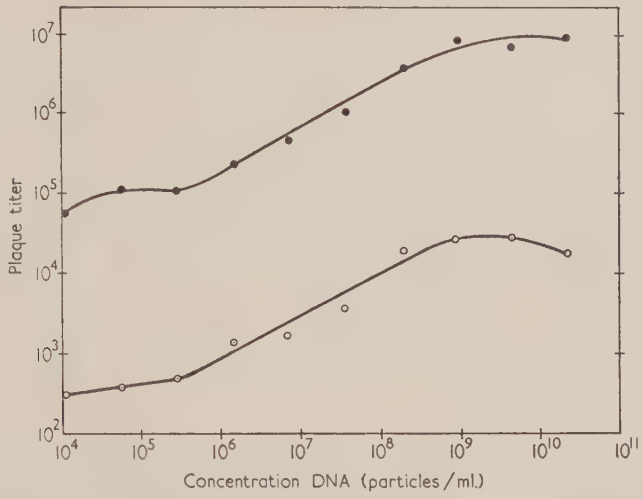

Fig. 5. Dilution curve (nonlinearity of assay). Curve A (⊙——⊙) indicates infective centers (plated after 10 min adsorption), curve B (●——●) represents mature phage produced after 2 hr incubation.

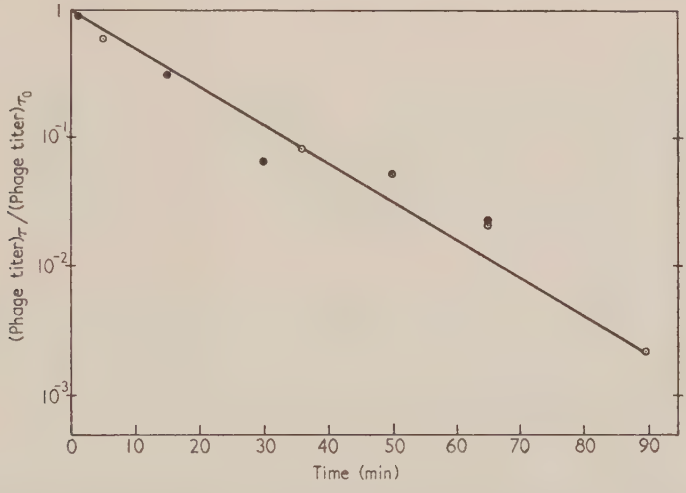

Fig. 6. DNase inactivation of CaϕX-DNA. The curve represents the normalized decline in final phage titer as a result of DNase inactivation of the infective particle. The actual inactivation is somewhat greater since the nonlinearity of assay tends to make lower concentrations of infective particles more efficient. Temperature 25°C, reaction mixture contained 0·005 M-$MgSO_4$, 0·1 M-tris pH 7·5, approx. 10^{13} CaϕX-DNA particles/ml. and 10^{11} DNase molecules/ml.

present in the infection mixture as free DNA, clearly indicating that new DNA is being made (rather than input DNA simply being "coated" with viral protein).

Reactions of DNase and trypsin with subviral particles: studies have been made of the actions of DNase and trypsin on CaϕX-DNA and HTϕX particles. Fig. 6 demonstrates that the action of DNase on CaϕX-DNA follows the kinetics of an enzymatic

inactivation of the particle. (These are the results of two different experiments and were not corrected for the nonlinearity of assay.) At a concentration of 1 enzyme molecule per DNA molecule the reaction is so rapid that it is impossible to measure the kinetics of inactivation. The same is true for the action of DNase on the HTϕX particle.

With trypsin, an inactivation of ϕX-DNA is observed which does not appear to be enzymatic. At molar concentrations of trypsin molecules comparable to or less than the molar concentration of the infecting particle, i.e. at "enzymatic" concentrations, there is no loss of infectivity, even with incubations up to 2 hours. However, at higher concentrations (approximately 10 or more enzyme molecules per infecting particle) there is a drastic loss of activity in the sample which is complete within 10 min. This is apparently the result of a stoichiometric binding of the basic protein, trypsin, to the DNA molecule. Additional evidence that ϕX-DNA is subject to non-enzymatic attachment to molecules such as trypsin may be obtained from "ring tests" similar to those used to test for antigen-antibody reactions. If a solution of ϕX-DNA is layered over a solution of trypsin a precipitate forms at the interface. On standing for 12 hr the "ring" remains, indicating that the binding between the molecules is strong.

Similar ring reactions are also observed with ϕX-DNA and lysozyme, with ϕX-DNA and anti-ϕX rabbit serum and with ϕX-DNA and normal (non-immune) rabbit serum. It thus appears that the highly charged polynucleotide probably combines readily with most basic proteins forming a "nucleoprotein" which appears to be biologically inactive.†

4. Discussion

From the results of the experiments it is clear that protoplasts of *E. coli* can be infected with the DNA of ϕX174. This DNA can be infective after it is separated from the protein coat or when, as with the HTϕX particle, it still has attached to it at least part of the viral protein. Using free ϕX-DNA, which has been purified by banding in the cesium chloride density gradient, the kinetics of infection of protoplasts by DNA can be studied in a manner similar to that used for the study of whole cells infected by virus.

The dilution experiments, however, suggest that "infection" is similar to the interaction of plant virus and their host plant cells. In particular, such dilution curves have been found for tobacco mosaic virus (TMV) infection (Kleczkowski (1950) reports dilution curves for TMV; Mundry & Gierer (1958) report data for TMV and TMV-RNA). The extent to which the kinetics of ϕX-DNA infection of protoplasts compare with those of TMV infection of sensitive leaves is not known. The results do seem to indicate that with this DNA, as with TMV and TMV-RNA, there is no specific attachment mechanism by which efficient infection can take place.

The stability of ϕX-DNA as compared to infectious TMV-RNA, the relative accuracy with which results can be reproduced with protoplasts, and the extensive background of information available on the growth of virus in bacteria suggest that this protoplast system has advantages for the investigation of viral replication initiated by free nucleic acid.

†BSA, at the proper concentration, also forms a precipitate with lysozyme. It is possible that BSA, in addition to stabilizing the protoplasts, serves to "protect" DNA in the adsorption mixture by binding lysozme and other basic molecules which might be present in the adsorption mixture due to the rupture of a few protoplasts.

The evident ability of these protoplasts to take up a single-stranded DNA suggests that bacterial transformation or infection with the DNA of other viruses might be achieved if the DNA employed were first converted to the single-stranded form (Doty, Marmur, Eigner & Schildkraut, 1960).

It is a pleasure to acknowledge the capable technical assistance of Miss Faith Poole. Several talks with Dr. Dean Fraser, Indiana University, about the preparation and use of protoplasts were of considerable value in developing the techniques for this research. Dr. Wolfgang Mundry's contributions in numerous discussions are gratefully acknowledged.

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Characterization of RNA Synthesized in *Escherichia coli* after Bacteriophage T2 Infection†

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The primary purpose of the present investigation was to obtain further information on the RNA synthesized in *E. coli* subsequent to infection by a virulent virus. Particular attention was focused on the physical nature of the RNA formed and its relation to the known particulate and soluble fractions which contain the bulk of the RNA in normal *E. coli* cells. To this end, examinations were carried out on extracts to permit identification of the ribonucleoprotein particles as well as on purified free RNA obtained from relevant fractions. The identification of newly synthesized RNA was accomplished by $^{32}PO_4$ labeling and the period studied was between 5 and 7 min after infection. Most of the experiments were performed with the T2 bacteriophage. The data obtained may be summarized as follows:

(1) Two categories of RNA are synthesized after infection with T2. One is found in the soluble and the other in the ribosomal fraction. The latter constitutes the bulk of the RNA formed in the period studied.

(2) Examination of the T2-specific ribosomal RNA reveals a number of features which distinguish it from the majority of the normal RNA. It exists mainly in the form of ribonucleoproteins possessing a sedimentation coefficient of 30 s. It is released as free RNA on dialysis against low Mg^{2+} concentration. After deproteinization, it was shown to have a sedimentation coefficient (about 8 s) which is considerably lower than the 25 s and 18 s values found for the two major components of ribosomal RNA. It also possessed a distinctly higher electrophoretic mobility. Finally, it is readily distinguishable from normal ribosome RNA in terms of its base composition, and thus appears to correspond to the newly-synthesized RNA studied by Volkin & Astrachan.

(3) In addition to the T2-specific ribosomal RNA a "soluble RNA" is synthesized after T2 infection. The T2-specific "soluble RNA" is similar to its normal counterpart in existing as free RNA and possessing the same average sedimentation coefficient. It is distinguishable by its higher electrophoretic mobility and markedly different base composition which resembles the T2-specific RNA found in the ribosomal fraction.

1. Introduction

Data accumulated over the past decade suggest that infection of bacterial cells with virulent viruses is followed by a restriction of protein and nucleic acid synthesis to a comparatively small class of molecules relevant to the formation of virus components. Thus, the infected bacterium-virus complex would appear to represent an attractive system for the experimental analysis of the relations amongst DNA, RNA, and protein (Crick, 1958; Hoagland, 1958).

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‡On leave from the Institute of Applied Microbiology, University of Tokyo, Japan.

Of particular immediate interest are the experiments of Volkin & Astrachan (1956*a*,*b*; Volkin, Astrachan & Countryman, 1958) who demonstrated the incorporation of ^{32}P into an RNA fraction which was distinguishable by its apparent base ratios from the bulk of the indigenous RNA. These ratios were determined by estimation of the relative ^{32}P content of the corresponding 2′,3′-nucleotides isolated from an alkaline hydrolysate of RNA from infected cells sampled after various periods of ^{32}P incorporation. The numbers so obtained appeared to mimic, during certain stages of infection, the DNA base ratio of the infecting particles.

The central importance of these observations made it desirable that they be extended in several directions. The existence of an RNA peculiar to virus infected cells is inferred from the Astrachan-Volkin experiments solely on the basis of the nucleotide labeling. It would clearly be of value to provide independent evidence in support of this inference. It would also be of interest to obtain information on the state of this RNA and its relation to the known RNA containing components of the cell.

It is the purpose of the present paper to describe experiments pertinent to this and other related problems. The data show that virus infection induces the formation of RNA components which can be separated from the bulk of the cellular RNA by centrifugation in a density gradient or by electrophoresis in starch columns. Two fractions specific to virus-infected *E. coli* have been identified. One, constituting the vast majority of the RNA synthesized, is a nucleoprotein sedimentable at 100,000 *g* consisting mainly of 30 s ribosomes. The other is a low molecular weight RNA found with, and corresponding in size to, the "soluble" RNA fraction. Both fractions are also distinguishable from normal components by their apparent base ratios.

2. Materials and Methods

(a) *Preparation of cells and conditions of infection*

E. coli strain B and bacteriophages T2 and T7 were used. Cells were grown overnight on a rotary shaker at 30°C in modified Penassay-broth (Bacto-beef extract 0·175%,

Table 1

Biological assays

Experiment no.	A	B
Phage used	T2	T7
Total bacteria	$2{\cdot}0\times10^{8}$/ml.	$2{\cdot}7\times10^{8}$/ml.
Number of infective centers	$1{\cdot}9\times10^{8}$/ml.	$3{\cdot}0\times10^{8}$/ml.‡
Average multiplicity† of infection	25	13
Uninfected bacteria	$< 1\times10/^{4}$ml.	$< 1\times10^{6}$/ml.
Ratio of uninfected bacteria to infected bacteria	$< 5\times10^{-5}$	$< 1{\cdot}9\times10^{-3}$

†Multiplicity $= \dfrac{\text{No. of phage added} - \text{No. of free phage}}{\text{No. of infected bacteria}}$

‡This figure was obtained without the use of anti-serum from the difference between total number of plaques and those due to free phage.

Bacto-yeast extract 0·15%, Bacto-peptone 0·5%, NaCl 0·35%, pH 7·4). The overnight culture was diluted 60-fold with fresh Penassay-broth and reincubated until a titer of 2 to 3×10^8 cells/ml. was attained. The bacteria were then harvested, washed with tris-(hydroxymethyl)aminomethane buffer (10^{-2} M, pH 7·3) containing KCl (0·05 M), and resuspended in a fresh Penassay-broth, at the original cell concentration. After temperature equilibration at 37°C the cells were infected with an input ratio of bacteriophage to bacteria of between 10 and 20. After 5 min, samples were taken and assayed for free phage, total plaque formers, and uninfected and infected bacteria by the procedures described by Adams (1959). In all experiments reported here, the number of uninfected bacteria was less than 2×10^{-3} of the number of infected bacteria. Typical assays involving infections with both T2 and T7 are presented in Table 1.

When T7 was used, phage infection and the subsequent ^{32}P pulse experiment were performed at 28°C instead of 37°C to extend the latent period as suggested by Volkin, Astrachan & Countryman (1958). Under these conditions, latent periods of 30 to 35 min and burst sizes between 100 and 150 were obtained.

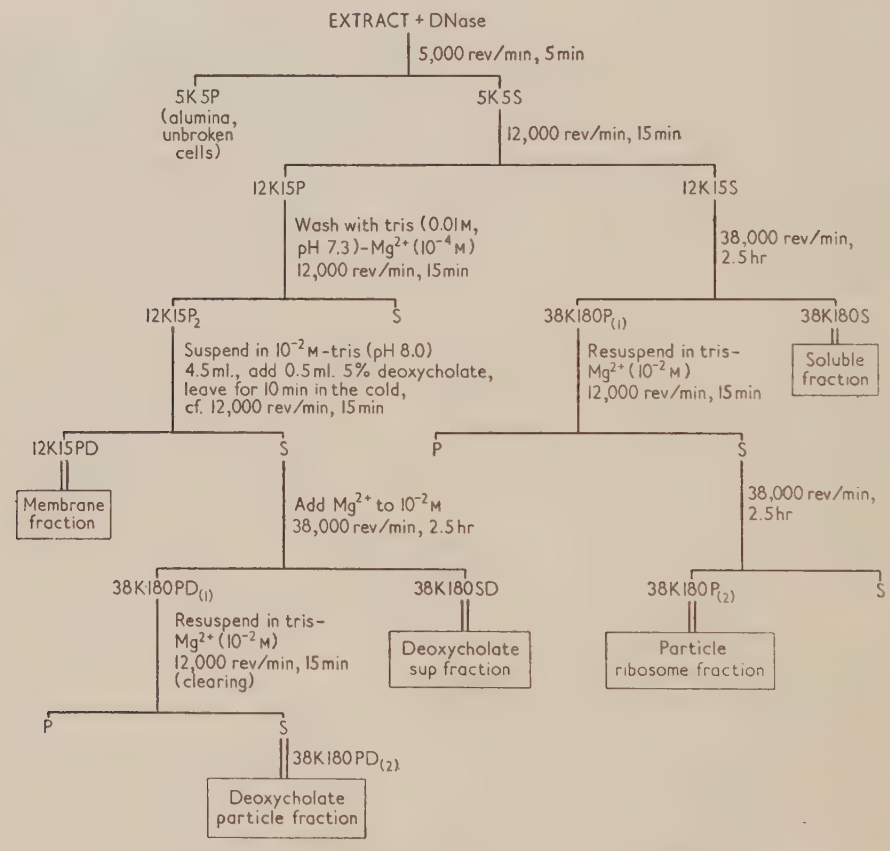

FIG. 1. Procedure for fractionation of *E. coli* cells.

The usual designations for centrifugal fractions are employed. Thus, 12K15P signifies a pellet derived from a spin at 12,000 rev/min for 15 min and 12K15S the corresponding supernate. With the machines employed 12K corresponds to 15,000 ***g*** and 38K to 1000,000 ***g***. The symbol D distinguishes fractions obtained from deoxycholate treatments.

(b) *^{32}P pulse experiment and the preparation and fractionation of cellular extracts*

^{32}P inorganic phosphate, obtained from the Oak Ridge National Laboratory, was neutralized and added to the culture of phage infected bacteria at 5 minutes after infection. Usually 2 min after ^{32}P addition (4 min at 28°C in the case of T7), NaN_3 was added to a

final concentration of 0·01 M to stop further assimilation of phosphorus, and immediately thereafter the whole culture was poured on to one third its volume of crushed ice made by freezing Penassay medium at − 70°C. The cells were centrifuged and washed twice with cold tris (0·01 M, pH 7·3)—KCl (0·05 M). The pellets were then ground with about 2·5 times their weight of alumina powder and extracted with approximately 5 vol of cold tris (0·01 M, pH 7·3)—$MgCl_2$ (10^{-2} M) solution containing deoxyribonuclease (DNase) (20μg/ml., crystalline material from Worthington Biochemical Corp.). Such conditions of extraction yield ribosomes mainly as 70 S particles which can be readily sedimented in a Spinco preparative ultracentrifuge (Tissières, Watson, Schlessinger & Hollingworth, 1959; Nomura & Watson, 1959). The subsequent fractionation of these extracts is detailed in the flow sheet of Fig. 1.

(c) *Method of preparation of RNA*

RNA was isolated from various fractions by the use of phenol (Gierer & Schramm, 1956). Samples were suspended or dissolved in 10^{-2} M-tris (pH 7·3) and treated first with sodium dodecyl sulfate (final concentration 0·2%) for 5 min at 20°C. All subsequent steps were performed in a cold room (5°C). After chilling, the solution was shaken with an equal volume of water-saturated, redistilled phenol for 8 min. After its separation from the phenol by centrifugation, the RNA-containing aqueous layer was removed and the treatment repeated twice with 0·5 vol aqueous phenol. The final aqueous layer was shaken with ether three times to remove most of the phenol. The ether was removed by bubbling nitrogen through the solution. The RNA solution thus obtained was dialyzed several times against tris (10^{-2} M, pH 7·3)—KCl (0·05 M) and finally against the buffers appropriate for subsequent analysis. The amount of ^{32}P in the dialysate was followed and dialysis was continued until the ^{32}P content of the dialysate became negligible.

(d) *Contamination with non-RNA phosphorus*

RNA preparations obtained by the procedure just described are not necessarily pure. This is particularly true for membrane fractions (See Fig. 1). An RNA preparation derived from membranes subsequent to a 2 min ^{32}P-pulse showed three major peaks of radioactivity on electrophoresis through starch columns. Of the total amount, only 5 to 10% of the activity was identifiable as RNA. As a result of such experiences, the absence of irrelevant ^{32}P containing material from each RNA fraction was examined by the electrophoretic technique. It was usually found that phenol-prepared RNA fractions from the ribosome and soluble components were quite free of contaminating phosphorus containing material. Virtually all of the ^{32}P contained in such preparations could be accounted for as RNA by procedures to be detailed below.

(e) *Purity of ribosome particles*

Similar care had to be exercised whenever it became desirable to fractionate ribosomes as intact particles as, for example, in density-gradient centrifugation. Particles prepared by the procedures described seem quite free of extraneous material by the usual analytical methods employed (Tissières *et al.*, 1959). However, when examined after a short ^{32}P-pulse experiment it was found that more than 90% of the ^{32}P was not associated with RNA. Whenever there were doubts concerning the purity of a labeled particle fraction, the following method was employed to ensure that the measurement referred to ^{32}P-labeled RNA. Both carrier albumin (1 to 5 mg/ml.) and RNA (1 to 5 mg of RNA prepared from *E. coli* by the phenol procedure) were added to the sample. To this solution, trichloroacetic acid (TCA) was added to a final concentration of 10%. The precipitate was washed twice and the RNA hydrolyzed by alkali (either with 0·33 N-KOH, 16 hr at 30°C, or with 0·1 N-NaOH, 2·5 hr at 100°C). The resulting solution was acidified to precipitate protein. The monoribonucleotide mixture in the supernatant was adsorbed on charcoal and washed three times with water containing a small amount of TCA. Finally, the charcoal, suspended in alcohol (50%)—NH_4OH (0·3% NH_3 by wt) solution, was transferred quantitatively to planchets and the radioactivity measured.

In some cases the purification was carried out with the aid of RNase. The washed TCA precipitate is dissolved in 0·2 M-Na acetate buffer and incubated with 20 to 50 mg/ml. of

RNase for 20 to 30 min at 37°C. The sample is then precipitated with TCA (10% final concentration) and the supernatant plated as "RNase solubilized counts."

(f) *Method of starch column electrophoresis*

Generally, the procedures used in this study are similar to those described in the papers by Rotman & Spiegelman (1954) and by Pardee, Paigen & Prestidge (1957).

Insoluble potato starch powder (E. H. Sargent and Co.) was washed with 0·05 N-NaOH several times. The alkali was removed by repeated washing with water, followed by several washings with the buffer to be employed in the electrophoresis. The standard buffer used was 0·03 M-tris (pH 7·8) which was supplemented with the indicated amounts of Mg^{2+} whenever ribosomes were run, to insure their physical integrity.

The column used was 3 cm wide, 1·5 cm high and 48 cm long. It was developed at 4°C for 8 to 11 hr at from 300 to 400 v with currents ranging from 6 to 10 mA. At the termination of the run, the surface of the starch column was blotted with a strip of filter paper and the approximate position of RNA was located on the paper strip under an ultraviolet lamp. The starch was then cut into 1 or 2 cm sections, depending on the position relative to the RNA region, and eluted with the buffer used for development. Both the amount of RNA and ^{32}P was measured on the eluate. Depending on the preparation, radioactivity was measured either directly or after subsequent purification by the procedures described in the preceding paragraph.

(g) *Method of sedimentation analysis using swinging bucket*

Ultracentrifugal characterization of RNA and ribonucleoprotein particles was carried out in a swinging bucket rotor, Spinco type SW-39, in a model L ultracentrifuge. Use of this rotor was advantageous because it provided convection-free sedimentation (as in an analytical ultracentrifuge) and because it allowed a convenient separation of the contents of the centrifuge tube into fractions at the end of the experiment.

At the beginning of each run, the RNA solutions were placed in the centrifuge tube in the form of a layer (volume = 0·4 ml.) on top of 4·4 ml. of an aqueous sucrose solution containing tris buffer, pH 7·8, at a concentration of 0·03 M. In this solution, there was a linear gradient of sucrose concentration ranging from 20% sucrose at the bottom of the tube to 3% sucrose at the beginning of the RNA layer. The sucrose gradient was obtained by filling the centrifuge tube from a mixing device having two cylindrical chambers (Britten & Roberts, 1960). One chamber contained 3% sucrose solution and the other contained the same volume of 20% sucrose solution. Tris buffer at a concentration of 0·03 M was present in both sucrose solutions. Within the RNA-containing layer, there was a sucrose gradient from 3% at the bottom to 0% at the top, and an inverse gradient of RNA concentration. These were produced by applying the layer from the mixing device, with 0·02 ml. of 3% sucrose solution in one side and 0·2 ml. of RNA solution (1 to 2 mg RNA per ml.) on the other.

After loading of the tube, the solution was centrifuged at 37,500 rev/min and 3°C for 8 hr in the case of RNA, or for a shorter time—3 to 5 hr in the case of ribonucleoprotein particles. At the end of this time, the centrifuge was stopped (without braking) and the rotor removed to a cold room. The tube was removed from the rotor and pierced at the lower end with a syringe needle. As the contents of the tube emptied through the needle, fractions of 10 drops apiece were collected. After suitable dilution of the fractions, the ultraviolet absorption at 260 mμ and RNA-^{32}P of each fraction were determined by the procedures described above. These data provide information on the distribution of total and labeled RNA along the centrifuge tube at the end of the run.

(h) *Calculation of the sedimentation coefficient of the ^{32}P-labeled RNA*

In the swinging bucket analysis of ribosome RNA from infected cells labeled with ^{32}P one observes a ^{32}P-labeled peak moving at a distinctly lower rate than the two main RNA components (see Fig. 3) which are known to have sedimentation coefficients of 18 s and 25 s. The position of these are readily identified by the u.v. absorption profile. While the sedimentation coefficient of the ^{32}P-containing component (S_{32_P}) could be guessed from its relative position, it seemed desirable to develop a method which would permit a more

accurate estimation. This would involve taking into account that the RNA molecules are being centrifuged through a medium in which both solvent density and viscosity vary with distance. The Appendix outlines the development of a relation which permits the calculation of $S_{^{32}P}$ from the sedimentation coefficient of the u.v. peak ($S_{u.v.}$) and the experimental ratio $S_{^{32}P}/S_{u.v.}$

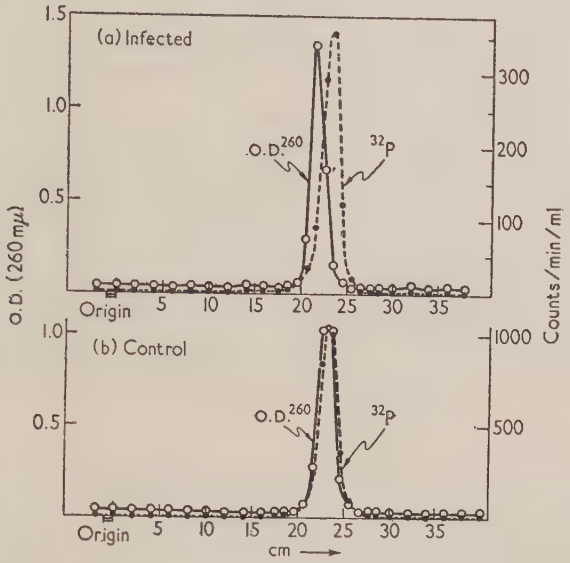

FIG. 2. Starch column electrophoresis of ^{32}P ribosome RNA.

(a) RNA from T2 infected cells, given ^{32}P between 5 and 7 min after infection.
(b) RNA from control cells given a 2 min ^{32}P pulse. In each case, electrophoresis was carried out for 10 hr at 8·0 v/cm, 6 mA in 0·03 M-tris buffer, pH 7·8.

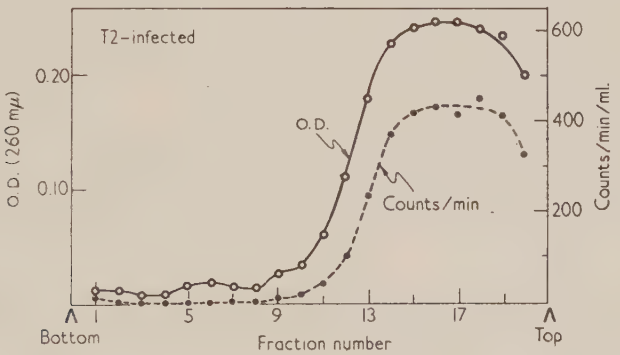

FIG. 3. Sedimentation diagram of ^{32}P-soluble RNA from T2 infected cells.

RNA prepared by phenol extractions of the 37K180S fraction of T2 infected cells exposed to ^{32}P between 5 and 7 min after infection.

0·2 ml. of ^{32}P soluble RNA solution was layered on 4·4 ml. of sucrose (concentration gradient from 3 to 20% sucrose), then centrifuged for 9·5 hr at 37,000 rev/min. Fractions containing 10 drops each were obtained by dripping from the lower end of the tube. All solutions contained 0·03 M-tris buffer at pH 7·8.

(i) *The isotope method of "base composition" analysis*

Astrachan & Volkin (1958) estimated the "base composition" of the RNA synthesized following phage infection by measuring the relative radioactivity associated with each of the relevant 2′ and 3′ nucleotides resulting from the alkaline hydrolysis of the RNA.

The numbers so obtained represent the true base composition of the newly formed RNA only if either of the following two assumptions are correct: (a) the specific activities of the 5′-nucleotide phosphorus atoms of the immediate RNA precursors are equal in the pool from which they are drawn; (b) the sequence of the bases along the RNA chain is random, for then the alkaline hydrolysis would tend to negate the effects of specific activity differences in the precursor nucleotide phosphates.

One cannot be certain that either one of the two conditions necessary for a rigorous deduction of base ratios from radioactivity distribution are in fact met. Nevertheless, it must be granted that differences in distributions observed between control and infected cells probably do reflect a real difference in the types of RNA synthesized in these two situations. The isotope method was, therefore, employed for estimating "apparent" base ratios. The details of the actual procedure may be briefly summarized.

To the dialyzed ^{32}P-RNA preparation, KOH was added to make a final concentration of 0·33 N and the mixture was incubated for 16 hr at 30°C. The resulting 2′,3′-nucleotides were separated using a Dowex-1 formate column (cross linking 8%, 1 × 5 cm). Eluants used were as follows: 0·05 N-formic acid to remove trace of nucleosides, 0·1 N-formic acid to elute cytidylic acid, and 1 N-formic acid to elute adenylic acid. A gradient from 2 N to 5 N-formic acid was used to collect guanylic and uridylic acids separately. Optical densities at 260 and 280 mμ, and radioactivity, were measured on each fraction. The amount of each nucleotide was calculated using the known molecular extinction coefficients.

3. Experimental Results

(a) *Distribution of RNA synthesized after T2 infection*

^{32}P-orthophosphate was added to the cells between 5 and 7 minutes after infection with bacteriophage T2. Table 2 lists the amounts of ^{32}P-RNA found at the end of this period in each of the components obtained from the fractionation described in Fig. 1. It can be seen that the bulk (63%) of the ^{32}P-RNA is found in the ribosome fraction [38K180P(2)].

Following removal of alumina and unbroken cells at 5000 rev/min, centrifugation of the extract at 12,000 rev/min yields a pellet fraction (12K15P(2) of Fig. 1) composed of membrane and wall fragments, which contains appreciable amounts of RNA. However, it was found (see section (d) below) that almost all of this was released in the form of 50 s and 30 s particles during treatment with deoxycholate. The residue (12K15PD of Fig. 1) contained little ^{32}P-RNA. The data suggest that under the conditions employed the bulk of the newly synthesized RNA was to be found in the ribosome fraction and, consequently, initial attention was focused primarily on the characterization of the ^{32}P-RNA of the fraction, (38K180P(2) of Fig. 1).

(b) *Characterization of "ribosome RNA" synthesized after bacteriophage T2 infection*

(i) *Starch column electrophoresis*

Orthophosphate-^{32}P was added at 5 min after T2 infection and the assimilation was stopped at 7 min and RNA prepared from the ribosome fraction as described under Methods. Fig. 2(*a*) illustrates typical profiles of radioactivity and optical densities at 260 mμ obtained when such preparations are subjected to starch column electrophoresis. Single, but non-coincident, peaks of u.v.-absorbing and ^{32}P-containing materials are observed, the ^{32}P material moving definitely faster than the bulk of the RNA. That the radioactivity peak is RNA, and not an irrelevant contaminating material, was established by the following experimental facts:—

1. More than 90% of the radioactivity was acid (10% TCA) insoluble;
2. After RNase treatment (crystalline pancreatic RNase 30 μg/ml., 37°C, 10 min) all the radioactive material was converted to an acid-soluble form;

3. No effect was observed when it was treated with DNase (crystalline pancreatic DNase, 30 μg/ml., 37°C, 10 min, in the presence of 10^{-3} M-Mg), whereas, under the same conditions, highly polymerized sperm DNA (250 μg/ml.) was completely hydrolyzed;
4. Finally, as detailed below, all the radioactivity contained in the initial peak was recovered as the corresponding ribonucleotides following alkaline hydrolysis and separation by ion exchange chromatography.

TABLE 2

Distribution of RNA synthesized in various cellular fractions after infection with T2

At 5 min after T2 infection 20 mc of $^{32}PO_4$ was added to 500 ml. of *E. coli* culture infected with T2. At 7 min after phage infection, Na azide was added and the whole culture was chilled with crushed frozen medium. Cells were collected and disrupted, and each fraction was obtained as described under Methods, except that washings of particle (38K180P(1) of Fig. 1) and cellular debris (12K15P of Fig. 1, 15 min centrifugation) were omitted to obtain a complete recovery of total RNA-^{32}P. Each fraction was then chemically fractionated by Hershey's (1953) modification of the Schmidt & Thannhauser procedure. The amount of RNA was determined from the optical density at 260 mμ of RNA fraction. RNA-^{32}P was calculated from the radioactivity of nucleotide obtained after column chromatography as described under Methods.

Experiment no.	1		2
Fractions	Total counts/min $\times 10^{-3}$	RNA-^{32}P % of total	Chemical amount % of total
(1) Particle ribosome fraction [38K180P(1)]	383	63	87
(2) Soluble fraction [38K180S]	49	8	6·5
(3) Deoxycholate supernatant fraction [38K180SD]	68	11	—
(4) Deoxycholate particle fraction [38K180PD]	94	15	—
(5) Membrane fraction [12K15PD]	15	3	1·5
(6) Total deoxycholate extracted, fractions [(3) + (4)]	162	26	5·1
(7) Total cellular debris, fractions [(3) + (4) + (5)]	177	29	6·6
Total RNA	609	100	100

It was concluded, therefore, that the material in the ^{32}P-peak was RNA and that it is distinguishable from the bulk of normal pre-existing ribosome RNA by its higher electrophoretic mobility.

It was of interest to look for a similarly labeled fraction in control preparations. Accordingly, non-infected cells were subjected to a 2 min ^{32}P-pulse subsequent to which the isolated RNA of the ribosomes was subjected to electrophoresis with results described in Fig. 2(*b*). It is evident that the u.v. absorption and radioactivity curves coincide quite well. The data suggest that infection with T2 leads to the preferential synthesis in the ribosomal fraction of RNA which has a mobility significantly higher than that which characterized normal RNA.

Volkin *et al.* (1958) have reported the formation of an RNA in the course of infection with T7 which is distinguishable from T2-specific RNA. One can calculate the base composition of the RNA formed from their specific activities, using the principle of isotope dilution, and the known composition of the ribosomal RNA which would be the principal unlabeled contaminant. Using their values for the latter one finds that the moles percent of cytidylic, adenylic, guanylic, and uridylic acids are respectively 24, 25, 28, and 23. Comparing these values with those found for normal *E. coli* RNA (see Table 3) indicates a rather good agreement. We need not here enter into a discussion of

Table 3

"Base composition" of ^{32}P ribosome RNA synthesized after T2 infection and base composition of cold ribosome RNA.

^{32}P was added during the time between 5 and 7 min after T2 infection. Both chemical amount and radioactivity were measured on the same sample. Two different ^{32}P RNA preparations, obtained from different pulse experiments, were analyzed and gave the same results (maximum difference is less than 10%). Data shown are the average of the two values. The numbers in parenthesis are taken from Astrachan & Volkin (1958).

	Mole per cent	
	^{32}P ribosome RNA	Cold ribosome RNA
Cytidylic acid	15·5 (17)	20·3 (23)
Adenylic acid	28·0 (31)	23·9 (23)
Guanylic acid	21·0 (20)	33·5 (31)
Uridylic acid	35·5 (31)	22·3 (23)

the significance of this comparison. For our present purposes we may merely note that the RNA formed during T7 infection is much closer in its base composition to normal *E. coli* RNA than is the variety synthesized in the course of infection with T2. This situation provides an excellent opportunity to test the meaningfulness of the separation of T2-specific RNA by zone-electrophoresis on starch columns. If the resolution is primarily due to base composition differences, the T7 variety of RNA should be difficult to separate from normal *E. coli* RNA. Accordingly, ^{32}P-RNA was prepared from the ribosome fraction of T7 infected *E. coli* cells which assimilated $^{32}PO_4$ at 28°C in the period between 5 and 9 min after infection. Its size was first determined by analysis of fractions obtained from a swinging bucket run and was found to be about the same as that of the T2-RNA. The peak of radioactivity corresponded to a sedimentation coefficient of about 8 s and very little radioactivity was associated with the two major RNA peaks corresponding to 25 s and 18 s components. Figure 8 shows the electrophoretic behaviour in a starch column. Here, we note relatively little difference in mobility between the unlabeled pre-existent RNA and the newly synthesized material. Since they are of the same size, the difference in mobility between the T2 and T7 specific RNA components may be a reflection of a difference in secondary structure (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959) which is a consequence of a difference in base composition.

(ii) *Sedimentation analysis*

Previous work (Bolton, Hoyer & Ritter, 1958; Tissières *et al.*, 1959) established that *E. coli* in log phase contains four varieties of ribosomes characterized by dif-

ferences in sedimentation coefficients (approximately 30 s, 50 s, 70 s, and 100 s). Tissières *et al.* (1959) express the interrelation amongst these particles by the following equation: $2(30\text{ s}) + 2(50\text{ s}) \rightleftharpoons 2(70\text{ s}) \rightleftharpoons 100\text{ s}$. The equilibrium position is strongly influenced by the concentration of Mg^{2+} (Bolton *et al.*, 1958; Tissières *et al.*, 1959; Spiegelman, 1958). The molecular weights of the 30 s and 50 s particles were found to be $1{\cdot}85\times10^6$ and $0{\cdot}95\times10^6$ respectively. The molecular weight of the 70 s ribosome is about $2{\cdot}8\times10^6$ and it can be dissociated at low Mg^{2+} levels into one 30 s and one 50 s particle.

Hall & Doty (1959) showed that calf-liver microsomes contain two categories of RNA molecules. One had a sedimentation coefficient of 28 s and the other of 18 s, corresponding to apparent molecular weights of $1{\cdot}3\times10^6$ and $6{\cdot}0\times10^5$ respectively. A very similar situation was found for the 70 s ribosomes which yield 25 s and 18 s components in the RNA prepared by the phenol method. The 18 s variety has a molecular weight of $6{\cdot}5\times10^5$ and that of the 25 s component was estimated to be $1{\cdot}2\times10^6$ (Hall, unpublished observations).

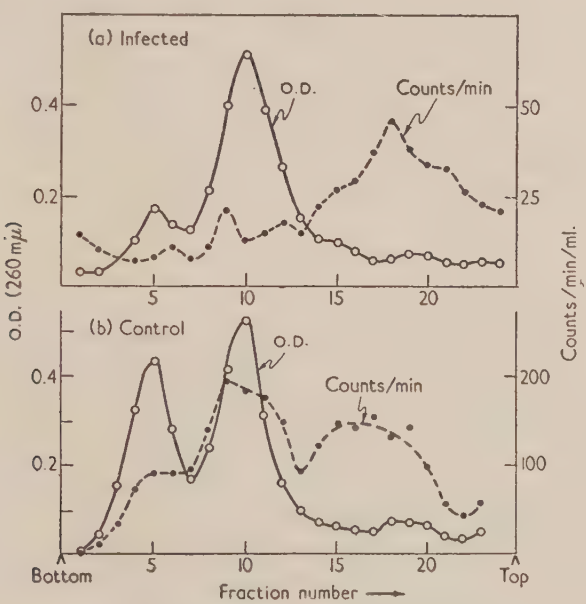

FIG. 4. Sedimentation of ^{32}P particle RNA.

(a) RNA from T2 infected cells, exposed to ^{32}P between 5 and 7 min after infection.

(b) RNA from the control cells given a 2-minute ^{32}P pulse. 0·25 mg RNA in 0·03 M-tris (pH 7·8) were layered on 4·4 ml. of sucrose solution, with a concentration gradient from 3 to 20%. This was centrifuged for 8 hr at 37,000 rev/min. Fractions were collected by piercing the lower end of the tube and collecting 10-drop fractions. To each fraction 1 ml. 0·03 M-tris buffer was added; optical density at 260 mμ and ^{32}P content were then measured.

In view of these findings, it was of obvious interest to determine whether the RNA preferentially synthesized following T2 infection falls into one of the known size categories.

RNA was prepared by the phenol method from the ribosome fraction of *E. coli* cells allowed to assimilate ^{32}P between 5 and 7 min after infection. It was then subjected to a swinging bucket sedimentation analysis in a sucrose gradient as described under Methods. It is seen from the results given in Fig. 4(a) that the main peak of ^{32}P-RNA sedimented at a considerably slower rate than did the pre-existing RNA.

From the relative positions of the peaks of ^{32}P-RNA and ultraviolet absorption at 260 mμ (corresponding to the 18 s and 25 s components of the pre-existing RNA), one can calculate the sedimentation coefficient of the major ^{32}P-RNA component (see Methods, section (h), and Appendix).

The sedimentation coefficient of this ^{32}P peak was 7·9 s when calculated on the basis of its motion relative to that of the 18 s peak, and 8·3 s calculated from the 21 s peak.

The average of these, 8·1 s, would correspond to a molecular weight of 1 to $1{\cdot}5 \times 10^5$ for the ^{32}P-RNA. This assumes that the configuration of the ^{32}P-RNA in solution is the same as that of microsomal RNA, for which $S^0 = KM^{1/2}$ (Hall & Doty, 1958).

It should be noted that in such experiments the samples contain unlabeled RNA which is recovered as high molecular weight polymer and serves as an internal control of artifacts of breakdown during the preparation. This serves to insure the meaningfulness of the lower sedimentation coefficient observed for the ^{32}P-RNA.

In a control experiment (see Fig. 4(b)) a sucrose gradient sedimentation analysis was performed with ribosome RNA prepared from uninfected cells after a 2-minute ^{32}P pulse. It will be noted that ^{32}P-RNA, possessing a sedimentation coefficient of about 10, was also observed in this case. However, the pattern of labeling is quite different from that obtained with the infected cells. In the control experiment, one finds the 18 s and 25 s components heavily labeled, whereas very little ^{32}P-RNA possessing these sedimentation coefficients is present in particle RNA derived from infected cells.

(iii) *"Base composition" analysis*

The bulk of the ^{32}P-RNA synthesized subsequent to phage infection is found in the ribosomal fraction when the latter is prepared by the methods described in the present paper. This did not obtain in the experiments of Volkin & Astrachan (1956*b*). It was of interest, therefore, to examine the apparent base ratios of the ^{32}P-RNA isolated by our procedures and to compare them with the data found by Astrachan & Volkin. The results are summarized in Table 3. The numbers in parentheses are taken from a comparable experiment by Astrachan & Volkin (1958). The agreement between the two sets of data is quite good, particularly in view of the known variation in molar ratios of labeled RNA observed in the course of the infection. It will be noted that in both cases the apparent content of adenylate and uridylate is higher in the ^{32}P-RNA than in the unlabeled fraction of the same cells. Further, the sum of the purines is equal to the pyrimidines in the RNA synthesized subsequent to virus infection, a characteristic not observed in the pre-existent RNA of the ribosomes.

(iv) *State of occurrence of T2-specific RNA*

From the experiments described above, it is clear that the major portion of the RNA synthesized after T2 infection is found in the ribosome fraction. This RNA differs from the pre-existent ribosomal RNA in several chemical and physical properties. It was of interest now to examine whether the T2-specific RNA is bound to protein, and if so, whether it is present in one of the nucleoprotein particles (100 s, 70 s, 50 s or 30 s) which comprise the bulk of the ribosome fraction. Two types of experiments which bear on this question have been performed; direct sedimentation analysis of the 12K15S (ribosomes + soluble) fraction, and starch column electrophoresis of the ribosome fraction at various Mg^{2+} concentrations.

(a) *Sedimentation analysis.* Figure 5(a) shows the results obtained by sucrose gradient sedimentation analysis of the 12K15S fraction of T2 infected cells and control cells. While appreciable amounts of ^{32}P were found associated with the 100 s, 70 s, and 50 s particles of phage-infected cells, it is clear that the major component in which phage-specific RNA is present has a sedimentation coefficient of 30 s. In the control experiment the pattern of ^{32}P incorporation was quite different. In uninfected cells (Fig. 5(b)) both the 30 s and 50 s particles contained considerable amounts of

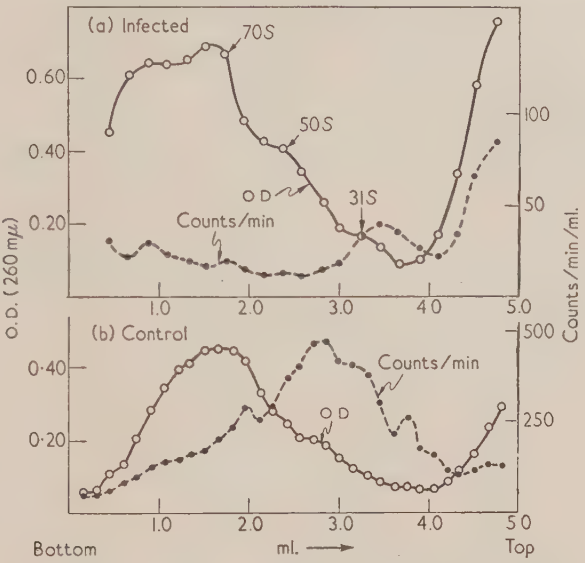

FIG. 5. Sedimentation analysis of the 12K15S (ribosomes and supernatant) fraction.

(a) 12K15S fraction from T2 infected cells given ^{32}P between 5 and 7 min after infection. Centrifuged for 140 min at 37,000 rev/min.

(b) 12K15S fraction from uninfected cells given a 2-min ^{32}P pulse. Centrifuged for 120 min at 37,000 rev/min. 0·2 ml. of the 12K15S fraction was layered on 4·4 ml. of sucrose solution (sucrose concentration gradient from 3 to 20%). All solutions contained 10^{-2} M-tris pH 7·3 and 10^{-2} M-$MgCl_2$. Upon completion of the run, fractions were collected by piercing the bottom of the tube with a needle; 10 drops comprise a fraction. After addition of 1 ml. buffer containing 10^{-2} M-$MgCl_2$, 10^{-2} M-tris, pH 7·3, the optical density at 260 mμ was measured. Each fraction was then precipitated with 10% TCA, washed twice and the ^{32}P solubilized by RNase was measured (see Methods).

^{32}P-RNA. A more rapid synthesis of RNA in the form of 30 s particles, or conversely a decrease in the rate of production of the larger particles, appears to be characteristic of phage infected *E. coli* cells.

(b) *Starch column electrophoresis.* These experiments were carried out to determine, under two sets of conditions, whether the electrophoretic properties of phage-specific RNA, as it occurred in the ribosome fraction prior to phenol extraction, were more nearly those of RNA, or those of ribonucleoprotein.

Ribosomes for this experiment were prepared from T2-infected *E. coli* which had assimilated $^{32}PO_4$ during the time between 5 and 7 min after infection. In this case, immediately after ^{32}P incorporation was stopped (by addition of azide), a concentrated suspension of carrier *E. coli* cells was added to 5×10^9 labeled, phage-infected cells. After washing of the cells and alumina grinding, ribosomes were prepared by the usual ultracentrifugation steps. Electrophoresis of the ribosomes was done under two sets of conditions:

CC*

1. After a 3 hr dialysis against 0·03 M-tris buffer, pH 7·8, containing 5×10^{-3} M-Mg^{2+};

2. After a 24 hr dialysis against 0·03 M-tris buffer, pH 7·8, containing 1×10^{-4} M-Mg^{2+}.

In each case, free RNA prepared from *E. coli* ribosomes by the phenol method was added to the sample just before electrophoresis was begun.

In the electrophoresis diagrams obtained, Figs. 6(a) and 6(b), mobility "reference

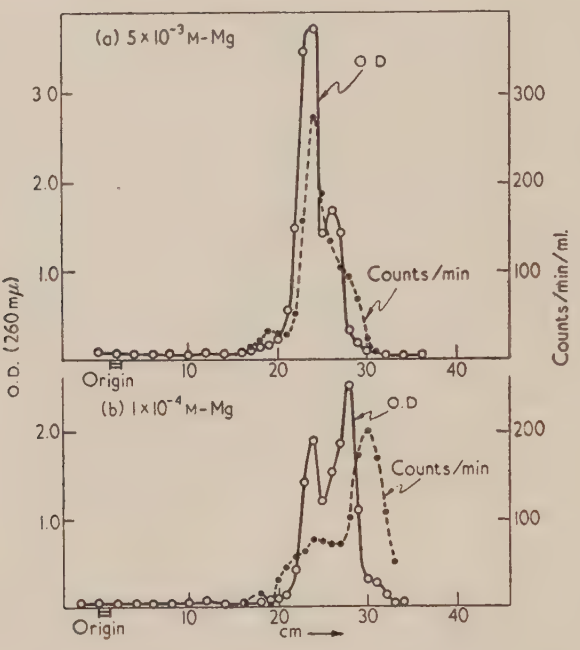

FIG. 6. Starch column electrophoresis of the ribosome fraction of T2-infected cells.

(a) Electrophoresis in 0·03 M-tris, pH 7·8 with 5×10^{-3} M-Mg^{2+} after 3 hr dialysis against this solvent. Duration of run—13 hr at 8·0 v/cm; 11 mA.

(b) Electrophoresis in 0·03 M-tris, pH 7·8, 1×10^{-4} M-Mg^{2+} after 24 hr dialysis against this solvent. Duration of run—13 hr at 6·0 v/cm; 4 mA.

Ribosomes used in this experiment came from T2-infected cells exposed to ^{32}P between 5 and 7 min after infection. *E. coli* particle RNA (prepared by the phenol method) was added as a marker just before electrophoresis. After elution of each fraction from starch (see Methods) OD at 260 mμ was measured; then the acid insoluble ^{32}P which was solubilized by RNase was counted (counts/min curve).

standards" are provided by the two peaks of ultraviolet absorption. The slower moving peak in each case contains the ribonucleoprotein particles, and the faster moving peak contains the added free RNA. From the position of the ^{32}P peak relative to the peak of ultraviolet absorption in each case, it is evident that:

(1) At concentrations of Mg^{2+} as high as 5×10^{-3} M, most of the T2-specific RNA (^{32}P-RNA) has the electrophoretic mobility characteristic of a nucleoprotein;

(2) When the Mg^{2+} concentration is reduced to 10^{-4} M by a 24 hr dialysis, the mobility of most of the ^{32}P-RNA is increased, becoming approximately equal to that of the T2-specific RNA obtained by phenol extraction of ribosomes (cf. Fig. 2(a)).

The conclusions derived from these sedimentation and electrophoresis experiments are the following: in the initial extract of the T2-infected cells (in 10^{-2} M-Mg^{2+}) T2-specific RNA is present in the form of a particle having a sedimentation coefficient

of approximately 30 s. The electrophoretic mobility of this particle (in 5×10^{-3} M-Mg^{2+}) would indicate that it is a ribonucleoprotein. When the Mg^{2+} concentration is lowered to 10^{-4} M, the presumed nucleoprotein is broken down liberating most of the T2-specific RNA as free RNA. The precise role of various experimental variables (length of dialysis, pH, Mg^{2+} concentration, total ionic strength, etc.) in causing this breakdown has not been established.

(c) *Characterization of "soluble RNA" synthesized after T2 infection*

Examination of the distribution of newly synthesized RNA, subsequent to infection, revealed that about 8% was to be found in the soluble fraction (Table 2). This fraction is known to contain free RNA of low molecular weight which has been implicated as a carrier of activated amino acids (Berg & Ofengand, 1958). In view of its presumed importance in protein synthesis, it was of interest to determine whether the newly synthesized soluble RNA was distinguishably different from that which exists in uninfected cells.

(i) *Starch column electrophoresis*

RNA was prepared by the phenol method from the soluble fraction (38K180S) derived from T2 infected cells subjected to a $^{32}PO_4$ pulse between 5 and 7 min sub-

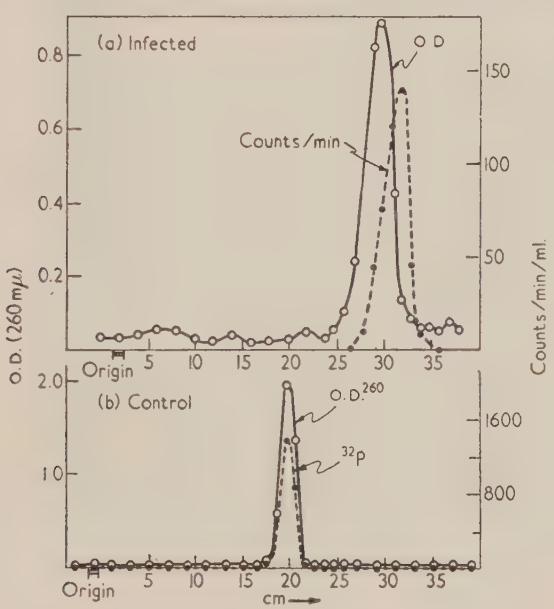

Fig. 7. Starch column electrophoresis of ^{32}P soluble RNA.

(a) Soluble RNA from T2 infected cells; ^{32}P incorporation occurred from 5 to 7 min after infection. Electrophoresis in 0·03 M-tris buffer, pH 7·8 for 9 hr at 7·5 v/cm; 7 mA.

(b) Soluble RNA from uninfected cells given a 2-min ^{32}P pulse. Electrophoresis for 10 hr at 6·0 v/cm at 12 mA.

Both soluble RNA preparations were made by phenol extraction of the 38K180S fraction.

sequent to infection. The resultant RNA was subjected to starch column electrophoresis the results of which are described in Fig. 7(a). It is evident that a single radioactive peak is observed corresponding to the newly synthesized RNA and that it has a distinctly higher mobility than the pre-existent unlabeled soluble RNA. This situation is to be compared with what is observed in the control experiment (Fig. 7(b)).

Here, uninfected cells were pulsed with ^{32}P under the same conditions and the purified soluble RNA was examined. No significant difference is detectable in this instance between the labeled and pre-existent RNA.

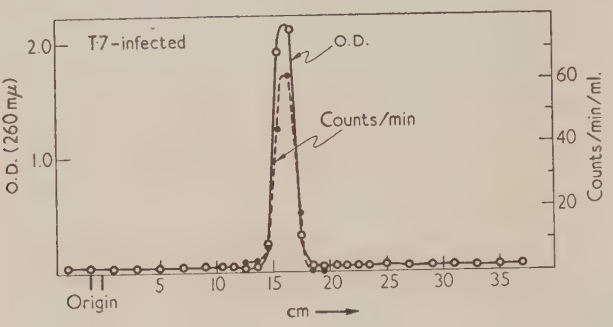

FIG. 8. Starch column electrophoresis of ^{32}P particle RNA of T7-infected cells.

RNA prepared by phenol extraction of ribosomes from T7 infected cells given ^{32}P from 5 to 7 min after infection.

Electrophoresis for 10 hr at 6·5 v/cm; 8 mA in 0·03 M-tris buffer (pH 7·8). Eluents of each fraction were precipitated with 10% TCA prior to counting of RNA-^{32}P.

(ii) *Sedimentation analysis*

Sucrose gradient sedimentation analysis was carried out with soluble RNA from T2-infected cells. RNA was prepared by the phenol method from the soluble fraction of cells exposed to ^{32}P between 5 and 7 min after infection. From the results (shown in Fig. 3) it is evident that the newly synthesized RNA has essentially the same sedimentation coefficient (about 4 s) as soluble RNA (Tissières, 1959) formed prior to infection, and is distinguishable in size from the ^{32}P-RNA synthesized in the ribosome fraction after T2 infection.

TABLE 4

Base composition of ^{32}P-soluble RNA synthesized after infection with T2 compared with normal soluble RNA

^{32}P was added during the time between 5 and 7 min after T2 infection. The base ratio of the normal S-RNA component was measured in both infected and uninfected controls by means of optical densities at 260 mμ of the appropriate fractions. The values are recorded in the "unlabeled" column as mean values of the two along with the average deviations from the means. The base ratio of the labeled soluble RNA formed after infection was determined by both isotope dilution and the distribution of total counts. The values given are the means and their average deviations.

	Moles per cent	
	^{32}P-labeled	Unlabeled
Cytidylic acid	16·2 ± 0·2	30·5 ± 0·6
Adenylic acid	27·1 ± 0·1	20·2 ± 0·3
Guanylic acid	21·1 ± 1·5	28·5 ± 0·1
Uridylic acid	35·1 ± 0·9	20·5 ± 0·5

(iii) *"Base composition" analysis*

Table 4 summarizes the results of a base composition analysis on a phenol purified preparation of soluble RNA derived from T2 infected cells. The molar ratios obtained

PLATE I. Analytical ultracentrifuge pattern of the deoxycholate particle [38K180PD(2)] fraction.

Rotor speed: 59,780 rev./min.
Interval between pictures: 2 min.
Solvent: 10^{-4} M-Mg^{2+}, 10^{-2} M-tris, pH 7·3
Ultraviolet absorption optics were used.
Measured sedimentation coefficients (S_{20}) for the two boundaries were 52 and 33 s.

[*To face page* 320

are to be compared with those observed in normal RNA contained in the soluble fraction. The composition of the unlabeled soluble RNA in infected cells is in excellent agreement with the values found for the same fraction in uninfected controls. It will be noted that the apparent base ratio of the RNA appearing in the soluble fraction after T2-infection is very different from that of normal soluble RNA. Comparison with the figures of Table 3 reveals that within experimental error the base ratios of the labelled RNA in infected cells is the same in both the ribosomal and soluble fraction.

(iv) *State of ^{32}P-"soluble RNA" synthesized after infection*

Previous experiments (Tissières, 1959; Nomura & Watson, 1959) suggest that the soluble RNA fraction of *E. coli* exists as free nucleic acid, and is not combined with either protein or other macromolecular components. It was of interest to determine whether this obtains also for the soluble RNA synthesized after T2 infection. To examine this the soluble fraction (38K180S) was prepared from T2 infected cells subjected to the usual 2-min ^{32}P pulse. This was dialyzed against tris buffer (10^{-2} M; pH 7·3) containing Mg^{2+} (5×10^{-3} M) and then fractionated electrophoretically on a starch column. Upon measurement of the ^{32}P-RNA and RNA content of each fraction a single peak of each was observed. The ^{32}P-peak moved slightly ahead of the RNA peak, as was the case with ^{32}P-soluble RNA prepared by the phenol method (Fig. 7(a)). Because the electrophoretic mobility of normal soluble RNA before phenol treatment is the same as the mobility of free RNA (Nomura & Watson, 1959) this result suggests that the ^{32}P-soluble RNA has the same mobility before and after phenol treatment. The data described are then consistent with the suggestion that the soluble RNA synthesized in a T2 infected cell exists in a free state like its normal counterpart.

In summary then, the experiments described in the present section suggest that, subsequent to T2 infection, a soluble RNA component is formed which is similar to normal soluble RNA in being uncombined and possessing approximately the same sedimentation coefficient. It is, however, distinguishable by its differing base composition and higher electrophoretic mobility.

(d) *^{32}P-RNA synthesized in other fractions*

As shown in Table 2, between 5 and 10% of the total RNA in the extract is usually found in the low speed pellet (12K15P of Fig. 1) which consists principally of cell walls and membranes. It was found that the bulk (85%) of the RNA in this fraction is released on exposure to deoxycholate. Plate I shows the sedimentation pattern of this material (as measured by ultraviolet absorption optics). The material studied in this run was the 38K180PD(2) fraction, resuspended in 10^{-4} M-Mg^{2+}, 10^{-2} M-tris, pH 7·3. Sedimentation coefficients (S_{20}) of 52 and 33 s were calculated for the two boundaries. Upon ultracentrifugal analysis, RNA prepared from this fraction by the phenol method exhibited the usual two components (25 s and 18 s) observed when ribosomal RNA is similarly analyzed. The available data suggest that a certain proportion of the ribosomes are rather tightly bound to some component of the low speed pellet. The fact that they are released as 50 s and 30 s particles does not imply the absence of the 70 s variety since the use of low levels of Mg during the deoxycholate extraction involves exposure of the membranes to a level of Mg^{2+} below 10^{-3} M. This treatment might have caused dissociation of 70 s particles.

Inspection of Table 2 reveals that ^{32}P appears in the membrane-bound RNA in the course of T2 infection. RNA prepared from the particles released by deoxycholate

was examined electrophoretically. The pattern of mobilities of labeled and unlabeled RNA was essentially the same as that observed with the RNA prepared from the ribosome (38K180P) fraction of the same cells.

It will be noted from Table 2 that somewhat in excess of 40% of the ^{32}P-RNA released by deoxycholate treatment is not sedimentable at speeds adequate to remove the particle fraction. This may very well be a consequence of the low Mg^{2+} level which attends the deoxycholate treatment. We have already noted (Figs. 6(a) and 6(b)) that low levels of Mg^{2+} result in release of ^{32}P-RNA as free nucleic acid from the ribosome fraction. RNA preparations, obtained from the deoxycholate-insoluble fractions of the membranes, were heavily contaminated with ^{32}P-labeled material which was not nucleic acid. A further investigation of the RNA of this fraction will require the development of better methods of purification.

4. Discussion

(a) *T2-specific RNA in the ribosome fraction*

Before undertaking an interpretation of the findings reported here it may be worth noting one difference between our data and those of Volkin & Astrachan (1956*b*). They found between 45 and 52% of the T2-specific RNA to be non-sedimentable at speeds adequate to pellet the ribosome fraction. Using the procedures described in the present investigation, one finds only about 10% of the labeled RNA remains in the supernatant fraction. This discrepancy may perhaps be explained by the comparatively low Mg levels (1×10^{-3} M) employed by Astrachan & Volkin in their extraction procedure particularly in view of our finding that the T2-specific ^{32}P-RNA is readily released from the ribosomal fraction as free RNA when the concentration of Mg^{2+} is reduced.

The experiments described in the present paper suggest that infection with T2 leads to the formation of at least two varieties of RNA. By the methods used for their localization, and because of their difference in size, one may be called "ribosomal" RNA and the other "soluble" RNA. The "ribosomal" RNA exists in a bound form, most likely as a ribonucleoprotein particle possessing a sedimentation coefficient of 30 s. The bulk of the newly synthesized RNA occurs in this form. After deproteinization, the principal component of the "ribosomal" RNA was found to have a sedimentation coefficient of about 8 s which is considerably less than the pre-existent major components of the ribosomal fraction which are 25 s and 18 s. The new RNA is further distinguishable from the normal cellular RNA by its higher electrophoretic mobility and difference in base ratios. A similar type of RNA molecule is synthesized subsequent to infection with T7. It is, however, evident that the T2 and T7 specific RNA molecules are readily differentiated by their electrophoretic mobilities and base ratios. This suggests that the preferential synthesis of this variety is not simply a generalized consequence of infection with virus but rather a specific response under the control of the incoming DNA.

There are several features of the present experiments and findings which would bear explicit emphasis. In the first place, the present study has been confined to the period between 5 and 7 min subsequent to infection. The distribution of label observed amongst the size varieties of RNA molecules is highly reproducible. However, the picture obtained may be uniquely characteristic of the particular period of infection chosen. The drift in base ratios towards normal *E. coli* RNA during the course of infection (Astrachan & Volkin, 1958) is already suggestive that late in the infection a

change in the specific control of RNA synthesis occurs. In any case, it is clearly necessary to subject other periods of the infection to the same sort of analysis employed in the present investigation to examine the period between 5 and 7 min.

It will be noted from Figs. 4(a) and 4(b) that the synthesis of ribosomal RNA of this smaller size (8 s) is not unique to the virus infected cell. A short ^{32}P-pulse experiment (Fig. 4(b)) readily reveals its existence in uninfected controls. It is evident that infection results essentially in the preferential synthesis of this size class during the period studied.

It is of interest to make an approximate estimate relevant to deciding whether the rate of protein synthesis is consistent with the supposition that the ribosomal RNA molecules formed during the period studied serve as templates for the protein molecules being formed.

A growing cell of *E. coli* contains 4×10^{-11} mg of RNA in its ribosome fraction. Since one 70 s ribosome contains 3×10^{-15} mg RNA, there are approximately $1{\cdot}3\times10^{4}$ 70 s ribonucleoprotein particles. Under the conditions employed in the present experiment, the doubling time is 30 min. A control non-infected cell would now synthesize 300 70 s particles (or 10^{-12} mg RNA) per min. The apparent specific activity of the ribosome fraction (i.e. the amount of ^{32}P-RNA per total amount of RNA) of phage infected cells was 25% that of control uninfected cells subjected to a similar 2-min pulse. Assuming that phage infection does not influence the specific activity of precursor material, we can now estimate the total amount of RNA synthesized between 5 and 7 min after infection as being 5×10^{-13} mg per cell infected. This corresponds to approximately 2×10^{3} RNA molecules of the 8 s variety. It might perhaps be argued that these are inactive subunits which must be assembled into the larger functional molecules characteristic of the 70 s ribosomes. This would still permit the synthesis of 170 of the latter in the 2 min interval. It should be noted that turnover of ^{32}P-RNA will tend to increase the actual rate of RNA synthesis and hence such calculations provide minimum estimates. Also, the calculation disregards RNA synthesized subsequent to infection but before or after the 2 min interval during which the cells are exposed to the isotope. One does not, of course, know how many ribonucleoprotein particles are necessary to make one kind of protein molecule, although presumably the minimum requirement would be one ribonucleoprotein particle for each different protein. The estimates made above would suggest that enough new RNA molecules are synthesized to take care of the needs generated by the requirement to form the relatively restricted variety of protein molecules relevant to virus production.

Such calculations tend only to show that the T2-specific RNA can satisfy the minimum requirements necessary to serve as specific guides for the synthesis of virus protein. It is obvious that considerably more must be done to provide more rigorous experimental support for this contention.

(b) *The soluble RNA fraction*

In addition to particle-bound RNA of high molecular weight, a variety of RNA appearing in the soluble fraction is also synthesized subsequent to T2 infection. The T2-specific soluble RNA has the same average sedimentation coefficient and is, therefore, probably in the same size range as normal soluble RNA. The fact that it possesses the same base ratio as the ribosomal fraction in the T2 infected cell suggests the obvious possibility that the "soluble" fraction may represent either breakdown products or precursor stages of the larger T2-specific RNA species. On the other hand,

it may serve the amino-acid accepting function of normal soluble RNA. In this connection a few facts are worth noting. The rate of protein synthesis in virus-infected cells proceeds at rates quite comparable to that characteristic of uninfected controls (Cohen, 1948). Nevertheless, this synthesis is not accompanied by a comparable metabolic activity in the normal soluble RNA fraction of the infected cell. From electrophoretic diagrams (see Figs. 6(a) and 6(b)) one can estimate that ^{32}P incorporation into the normal soluble RNA fraction is less than 2% of that observed in uninfected controls. The questions raised here can best be resolved by a direct investigation of the functional capacities of newly synthesized "soluble RNA" in phage infected *E. coli*.

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Appendix

Calculation of Sedimentation Coefficients in Density and Viscosity Gradients

For a macromolecule sedimenting in a centrifugal field, the sedimentation coefficient is defined as $S = \frac{dx/dt}{\omega^2 x}$, where x is the radial distance of the macromolecule from the center of rotation, dx/dt is the velocity of sedimentation, and ω is the angular velocity of the centrifuge rotor. If two banks or boundaries corresponding to two different

types of macromolecules A and B, present in the same solution, begin sedimenting from a common point x_0 at time O and sediment to points x_A and x_B at time t, the ratio of S_A to S_B is readily calculated.

$$S\omega^2 dt = \frac{dx}{x} \tag{1}$$

$$S_A \int_0^t \omega^2 dt = \int_{x_0}^{x_A} \frac{dx}{x} \tag{2a}$$

$$S_B \int_0^t \omega^2 dt = \int_{x_0}^{x_B} \frac{dx}{x} \tag{2b}$$

Since both A and B sedimented in the same rotor during the same time interval, $\int_0^t \omega^2 dt$ has the same value in (2a) and (2b); therefore,

$$\frac{S_A}{S_B} = \frac{\int_{x_0}^{x_A} \frac{dx}{x}}{\int_{x_0}^{x_B} \frac{dx}{x}} = \frac{\ln \frac{x_A}{x_0}}{\ln \frac{x_B}{x_0}} \tag{3}$$

In the case of sedimentation in a sucrose gradient, the calculation is complicated because the solvent density and viscosity are not uniform throughout the length of the centrifuge tube. The ultraviolet-absorbing component, having sedimented further into the sucrose gradient, would have been retarded relatively more than was the ^{32}P containing component. In correcting for this effect, corrections are applied both to S_{uv} and to $S_{^{32}P}$ in order to obtain S^0_{uv} and $S^0_{^{32}P}$, the values in a reference solvent, which we shall take as water at 5°C. To correct the observed S, we use:

$$S^0 = S\left(\frac{\eta}{\eta^0}\right)\frac{1 - \bar{v}\rho^0}{1 - \bar{v}\rho} \tag{4}$$

in which η is the solvent viscosity, ρ is the solvent density, η_0 and ρ_0 refer to the reference solvent, and $\bar{v}$ is the partial specific volume of RNA. By combining (1) and (4) and rearranging:

$$S^0\omega^2 dt\left(\frac{\eta^0}{1 - \bar{v}\rho^0}\right) = \frac{dx}{x}\left(\frac{\eta}{1 - \bar{v}\rho}\right) \tag{5}$$

If sedimentation is taking place in a sucrose gradient, both η and ρ vary with x.

$$S^0\omega^2 dt\left(\frac{\eta^0}{1 - \bar{v}\rho^0}\right) = \frac{dx}{x}\left(\frac{\eta(x)}{1 - \bar{v}\rho(x)}\right) \tag{6}$$

Equation (6) in integrated form describes the sedimentation of the u.v. and ^{32}P components during the swinging bucket run.

$$\begin{aligned} S^0_{uv}\left[\frac{\eta^0}{1 - \bar{v}\rho^0}\int_0^t \omega^2 dt\right] &= \int_{x^0_{uv}}^{x^t_{uv}} \frac{\eta(x)}{1 - \bar{v}\rho(x)} \cdot \frac{dx}{x} \\ S^0_{^{32}P}\left[\frac{\eta^0}{1 - \bar{v}\rho^0}\int_0^t \omega^2 dt\right] &= \int_{x^0_{^{32}P}}^{x^t_{^{32}P}} \frac{\eta(x)}{1 - \bar{v}\rho(x)} \cdot \frac{dx}{x} \end{aligned} \tag{7}$$

The quantities within brackets have the same value in each of the equations above; therefore,

$$\frac{S^0_{uv}}{S^0_{^{32}P}} = \frac{\int_{x^0_{uv}}^{x^t_{uv}} \frac{\eta(x)}{1 - \bar{v}\rho(x)} \cdot \frac{dx}{x}}{\int_{x^0_{^{32}P}}^{x^t_{^{32}P}} \frac{\eta(x)}{1 - \bar{v}\rho(x)} \cdot \frac{dx}{x}} \tag{8}$$

To a very close approximation, the viscosity and density of aqueous sucrose solutions at 5°C are given by:

$$\eta = \eta^0 (1 + \cdot 034c)$$

and

$$\rho = \rho^0(1 + \cdot 0041c)$$

thus

$$\left(\frac{d\eta}{dx}\right) = \cdot 034\eta^0, \quad \left(\frac{d\rho}{dc}\right) = \cdot 0041\rho^0 \tag{9}$$

Because of the linear dependence of viscosity and density upon sucrose concentration and because the sucrose gradient is linear,

$$\begin{aligned} \eta(x) &= \eta(x^0) + \left(\frac{d\eta}{dx}\right)(x - x^0) \\ \rho(x) &= \rho(x^0) + \left(\frac{d\rho}{dx}\right)(x - x^0) \end{aligned} \tag{10}$$

$$\left(\frac{d\eta}{dx}\right) = \left(\frac{d\eta}{dc}\right)\left(\frac{dc}{dx}\right), \quad \left(\frac{d\rho}{dx}\right) = \left(\frac{d\rho}{dc}\right)\left(\frac{dc}{dx}\right) \tag{11}$$

The ratio $\frac{S^0_{uv}}{S^0_{32P}}$ can thus be written as,

$$\frac{S^0_{uv}}{S^0_{32P}} = \frac{\eta_0 \int_{x^0}^{x^t_{uv}} \frac{1 + (x - x^0)\left(\frac{d\eta}{dx}\right)}{1 - \bar{v}\rho^0\left[1 + (x - x^0)\left(\frac{d\rho}{dx}\right)\right]} \cdot \frac{dx}{x}}{\eta^0 \int_{x^0}^{x^t_{32P}} \frac{1 + (x - x^0)\left(\frac{d\eta}{dx}\right)}{1 - \bar{v}\rho^0\left[1 + (x - x^0)\left(\frac{d\rho}{dx}\right)\right]} \cdot \frac{dx}{x}} \tag{12}$$

The definite integrals in equation (12) are of the form

$$\eta_0\left[\int_{x^0}^{x^t} \frac{a}{c + kx}\,dx + \int_{x^0}^{x^t} \frac{b}{x(c + kx)}\,dx\right] = \eta_0\left[\frac{a}{k}\ln(c + kx) + \frac{b}{c}\ln\left(\frac{x}{c + kx}\right)\right]_{x^0}^{x^t} \tag{13}$$

where

$$\begin{aligned} a &= \frac{d\eta}{dx} \\ b &= 1 - \left(\frac{d\eta}{dx}\right)x_0 \\ c &= 1 - \bar{v}\rho_0 + \left(\frac{d\rho}{dx}\right)\bar{v}\rho_0\, x_0 \\ k &= -\left(\frac{d\rho}{dx}\right)\bar{v}\rho_0 \end{aligned}$$

From the respective positions in the swinging bucket rotor of the fractions containing the maximum amounts of ^{32}P and ultraviolet absorption, together with the known dimensions of the rotor and centrifuge tube, values for x^t_{32P} and x^t_{uv} were obtained. Substitution of numerical values for these and the constants a, b, c, k, and x_0 followed by integration yielded S^0_{uv}/S^0_{32P}. The gradient of sucrose concentration (dc/dx) initially in the tube was used in evaluating $\left(\frac{d\eta}{dx}\right)$ and $\left(\frac{d\rho}{dx}\right)$ from (11).

The authors would like to express their thanks to Miss Therese Ming-Chung Mar for her skilful and enthusiastic assistance in the performance of the experiments described.

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Letter to the Editor

Discrete Low-Angle X-Ray Diffraction from Air-Dried Mitochondria

An intense low-angle X-ray diffraction pattern has been observed from air-dried insect flight muscle. This X-ray diffraction pattern is unlikely to arise from the muscle filaments because previous work on striated muscle has shown that muscle diffracts quite weakly both in the native or wet state and after air-drying. Insect flight muscle (Watanabe & Williams, 1951; Pringle, 1957) contains numerous large mitochondria or sarcosomes which occupy about one third of the volume of the fibre. Electron microscopy (Robertson, 1959; Bennett, 1960) has shown mitochondria to consist of an outer double membrane enclosing a series of transverse double membranes or cristae. The mitochondria lie in rows between the muscle myofibrils and the cristae are in planes perpendicular to the muscle filaments. The observed X-ray diffraction pattern is interpreted as arising from the regular packing of the cristae and the large period corresponds to the crista-to-crista distance in the mitochrondria.

The X-ray specimens which have been studied are the indirect flight muscles of the blowfly *Calliphora* (both the dorsal longitudinal and the dorsoventral muscles) and the indirect flight muscles of the waterbeetle *Dytiscus*. The best orientation was obtained when the muscles were air-dried before dissection and then the X-ray diffraction pattern consisted of a series of meridional arcs. It follows that the large period lies along the fibre axis direction which is coincident with the muscle filaments. For untreated air-dried specimens up to 8 orders of the large period $d = 300 \pm 5$ Å have been recorded. It should be noted that this spacing cannot be identified with the diffraction pattern of striated muscle fibres, for both the myosin period of 435 Å and the actin period of 410 Å remain unchanged even after drying (Worthington, 1959); and in any case this pattern is much weaker than that observed here.

A typical photograph of the X-ray diffraction for air-dried flight muscle of the blowfly *Calliphora* is shown in Plate I. The exposure time was 1 day and the first, second, third and fourth orders of diffraction were very strong, weak, medium and very weak respectively. The photograph in Plate II had less exposure and the first order is shown more clearly. The X-ray camera (Worthington, 1959) had a specimen-to-film distance of 6·7 cm in all experiments.

For the interpretation of the X-ray data in terms of some model the X-ray reflections were considered to arise from a one-dimensional array of cristae. The cristae have been identified with a unit membrane structure (Robertson, 1959) consisting of a bimolecular layer of lipid of thickness l with the outer surfaces covered by a layer of non-lipid or protein material of thickness p. A survey of the electron micrographs in the literature (Robertson, 1959; Bennett, 1960) was made to estimate l and p as fractions of the large period d. The fractions were fairly constant although the absolute values varied considerably. To measure the large period as defined here a section perpendicular to the planes of the cristae is required. The values $l = d/4$ and $p = d/8$ were obtained and electron densities of 1 unit and 4 units respectively greater than the electron density of the interspace were assigned. The model is illustrated in Fig. 1.

The integrated intensity $I(n)$ of the nth order X-ray diffraction can be written

$$I(n) \propto 1/n^2 [4 \sin 2 \pi n(\tfrac{1}{4}) - \sin 2 \pi n(\tfrac{1}{8})]^2$$

giving $$I(1) \approx 43, I(2) \approx 1, I(3) \approx 5, I(4) = 0$$

This agrees with the observed intensity variation of very strong, weak, medium and very weak. The X-ray analysis alone does not provide a unique solution, but the fact that the model is in agreement with both electron micrographs and the X-ray data suggests that the model, though simplified, is essentially correct.

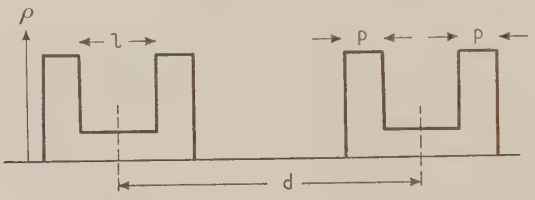

FIG. 1. Model of cristae showing the variation of electron density ρ in a direction perpendicular to the cristae surfaces.

The muscle was treated with various solvents before air-drying and any change in the X-ray diffraction pattern recorded. The initial aim was to break down the lipoprotein complex by the use of lipid solvents. Other solvents have also been used and the effect of all solvents studied so far, for treatment not exceeding 1 day, was to increase the large period. Some results are presented in Table 1; in each case the first

TABLE 1

Solvent	Time	Macroperiod
butanol	1 hr	310 Å
0·09 M-NaCl	1·5 hr	315 Å
water	1 day	340 Å
15% acetone in water	1 day	340 Å
30% acetone in water	1 day	370 Å

three orders of diffraction were observed. No systematic study has been made of the change in the large period with different solvents as a function of time. However, if the treatment is prolonged, the intensity of the diffraction diminishes until only the first order is observable and, for treatments exceeding 3 to 4 days, even this disappears. Frequently, when the meridional diffraction has vanished a moderate-angle X-ray diffraction may appear, usually as a ring at 47 Å, but on occasions, as an equatorial arc. The best example is shown in Plate III with the first three orders of 47 Å present on the equator. The specimen was the indirect flight muscle from the waterbeetle *Dytiscus* and the exposure time was 1 day. The appearance of this new diffraction suggests that, after the lipoprotein complex has been broken down, the lipid crystallizes out as a separate phase. The 47 Å spacing is not characteristic of insect flight muscle as this spacing has been observed quite often in the study of other muscles. Nevertheless, the 47 Å equatorial spacing is frequently observed after the meridional low-angle pattern has disappeared and the patterns rarely occur together.

All the muscles which gave the low-angle X-ray diffraction pattern (Plates I and II) have a characteristic colour of yellow to brown whereas the leg muscles of *Dytiscus*

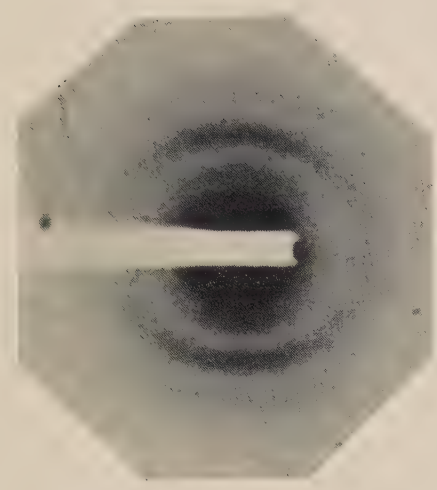

PLATE I. Diffraction pattern of untreated air-dried muscle.

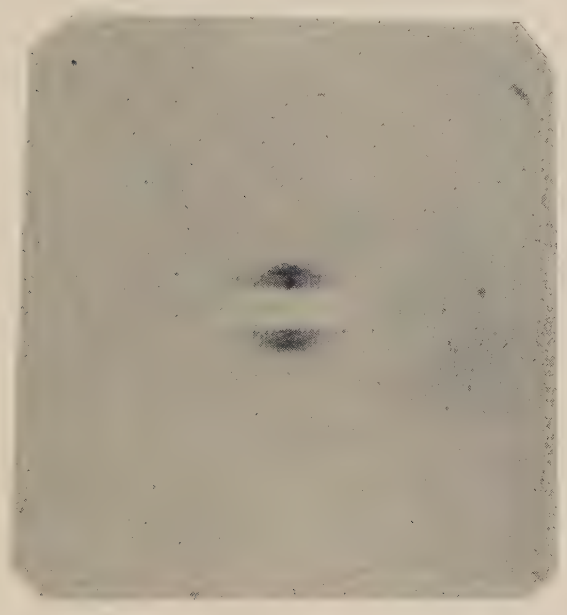

PLATE II. Diffraction pattern of untreated air-dried muscle, less exposure than Plate I.

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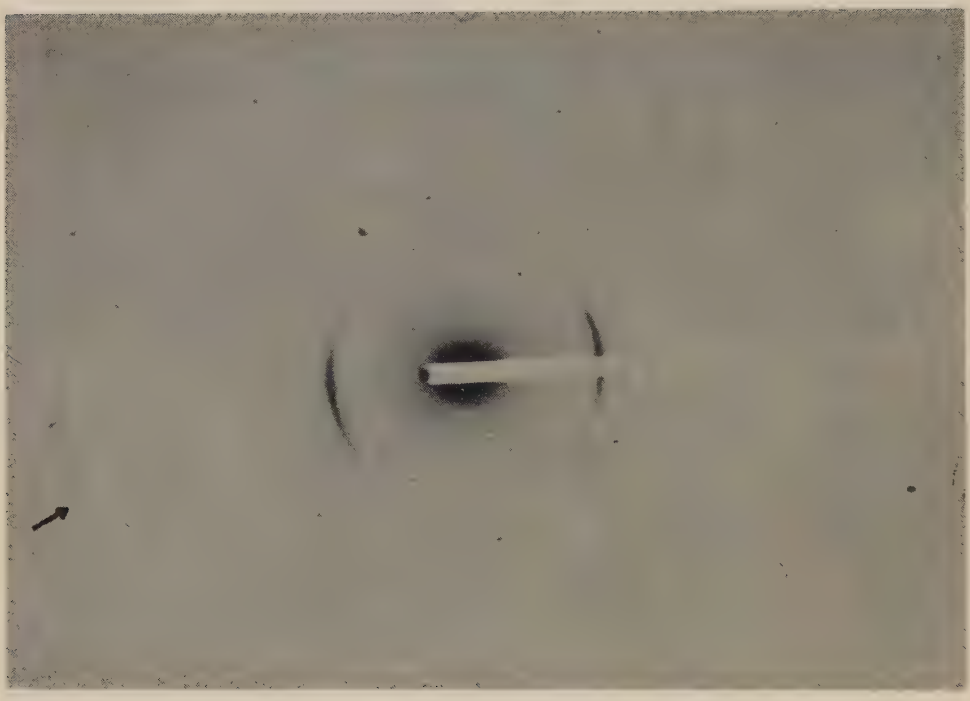

PLATE III. Diffraction pattern of air-dried muscle after prolonged treatment.

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are white. A number of workers (Pringle, 1957) have reported that the leg muscles of insects have much smaller mitochondria than the flight muscles. A spectrophotometric study (Nakatsugawa, 1960) of the red and white muscles of the cockroach *Periplaneta americana L* has shown that the red muscles contain a greater quantity of cytochrome than the white muscles. This difference in cytochrome content was considered to be due to the presence of more mitrochondria in the red muscles. It follows that the low-angle X-ray diffraction pattern is not likely to be obtained from the white muscles. In agreement with this expectation the low-angle pattern has not been observed with air-dried leg muscles of *Dytiscus* although the molecular filaments, myosin and actin, are packed in the same hexagonal lattice $a = 560$ Å as the flight muscles (Worthington, 1960).

The experimental observations are consistent with the interpretation that the meridional low-angle X-ray diffraction pattern comes from the mitochondria in insect flight muscle. Although experiments involving isolated mitochondria might provide direct evidence, it is doubtful if order would be maintained during and after isolation, whereas in the present study the mitochondria are held in position by the muscle myofibrils. A common source of mitochondria in other studies, e.g. electron microscopy (Robertson, 1959), has been the liver of animals. Preliminary X-ray work on such specimens, untreated and in the native state, gave no X-ray diffraction whereas on air-drying one or more lipid reflections were observed. The following spacings were obtained from beef liver, 45 Å, 52 Å and 63 Å; rat liver, 47 Å; mouse liver, 42 Å. The X-ray patterns from liver are similar to that shown in Plate III except that the X-ray reflections generally show random orientation.

It is of interest that no diffraction has been observed in the native state. This observation shows that the cristae are not ordered in the native state but regular order is only achieved after air-drying.

I wish to thank Professor J. T. Randall for provision of facilities, Dr. J. Hanson for suggesting work on insect flight muscle and Dr. M. H. F. Wilkins for helpful advice.

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